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SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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STUDIES ON BACTERIAL ENZYMES.

IV. The Maltase and Lipase of the Botulinus Bacillus.

By JAMES M. NEILL, Ph.D., and WILLIAM L. FLEMING.

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(Received for publication, March 4, 1927.)

INTRODUCTION.

The present paper reports a study of the maltase and lipase derived from *botulinus* bacilli; the activity of the enzyme was demonstrated with sterile solutions of the bacterial cells not only devoid of living bacteria but freed from cell fragments by Berkefeld filtration. The investigation was undertaken as a step toward obtaining further knowledge of the mechanism of metabolism of this toxin-forming species of bacterium. The fact that the *botulinus* bacillus is an anaerobe lends more general interest to a study of its enzymes since relatively little work has been done on the enzymes of anaerobic organisms.

Although maltase and lipase derived from different bacteria have been reported by a number of workers, no previous demonstration of these enzymes from *botulinus* bacilli appear in the literature. Several reports (1) have been made of the proteolytic action of sterile fluids obtained from broth cultures of *botulinus* bacilli; this work has been done with supernatant fluids of broth cultures. The sterile bacterial solutions used in the present study differ from the preparations of previous workers in that they contain the intracellular substances liberated when the bacterial cells are disintegrated in addition to the soluble products of growth.

EXPERIMENTAL.

Methods.

Preparation of Bacterial Extracts, or Enzyme Solutions.—The bacterial extract, or enzyme solution, was prepared from a representative, toxin-producing strain of *botulinus* bacilli (Type A) furnished by Dr. Jacques Bronfenbrenner of The Rockefeller Institute for Medical Research.

Large Florence flasks were filled aseptically with sterile meat infusion broth,

placed in the Arnold sterilizer, and steamed for 1 hour to drive out dissolved air; a heavy layer of sterile vaseline was then poured over the hot broth; the culture medium extended into the neck of the flasks so that only a small surface of the fluid required the vaseline seal for protection from air. This air-free medium was inoculated with a young culture of *botulinus* bacilli and incubated at 35°C. for 15 hours. The broth culture was then distributed into sterile, 250 cc. centrifuge bottles, sealed with vaseline, and centrifuged. The supernatant was removed and the sedimented bacilli from each 250 cc. bottle were suspended in about 5 cc. of the supernatant fluid. (The bacilli were suspended in the supernatant culture fluid because we believe that the presence of certain constituents of the broth either facilitates the subsequent dissolution of the bacteria or renders the liberated bacterial substances more soluble.)

The bacterial suspension was placed in long, narrow Pyrex tubes, sealed with vaseline, and repeatedly frozen and thawed as in the preparation of sterile pneumococcus (2) and meningococcus (3) extracts. The *botulinus* bacilli do not disintegrate easily as do pneumococci and meningococci and it was necessary to subject the suspension to about 100 repeated freezings and thawings. After this treatment, microscopic examination of the *botulinus* suspension indicated that the bacilli were well broken up, since the prepared films consisted almost entirely of bacterial detritus with only an occasional, definitely outlined bacillus. The suspension of disintegrated bacilli was centrifuged at high speed for 1 hour and the supernatant fluid was filtered through a Berkefeld candle. The filtered solution was distributed into long narrow test-tubes, sealed with vaseline, and stored at 2°C.

The sterile filtrate obtained represents an extract of the intracellular substances liberated when *botulinus* bacilli are disintegrated by repeated freezing and thawing. Since the bacilli were originally suspended in small portions of the supernatant culture fluid, these extracts contain broth and the soluble products liberated into the culture fluid during early growth of the culture, in addition to the intracellular substances derived by dissolution of the bacteria. After Berkefeld filtration, the extracts of the *botulinus* bacilli are perfectly clear and are indistinguishable in appearance from uninoculated broth. The presence of a considerable amount of dissolved protein material derived from the disintegrated bacterial cells is indicated by the heavy protein precipitation which occurs when these clear solutions are heated.

These sterile extracts of *botulinus* bacilli should contain both the exocellular and endocellular enzymes of the *botulinus* bacillus, provided that the enzymes are neither destroyed during the preparation processes nor held back by the Berkefeld filter. The term "enzyme solution," when used in the following text, refers to the bacterial extracts described above.

Sterility Controls.—After filtration, the sterility of the enzyme solution itself was proved by cultures in glucose broth. No antiseptics were added to any of the hydrolysis mixtures, since the sterility of each mixture was controlled by cultural methods.

Demonstration of the Hydrolysis of Maltose by Botulinus Enzymes.

The carbohydrate-fermenting proclivities of *botulinus* bacilli are not pronounced. While glucose is commonly fermented, there is considerable dispute in the literature concerning their fermentation of the common disaccharides, due largely to the fact that different strains of *botulinus* vary in their disaccharide fermentation. Living cultures of the strain used in this investigation fermented maltose rather slowly, and seemed completely devoid of the property of producing either acid or gas from lactose or sucrose. These fermentation properties, while not agreeing with all reports in the literature, are possessed by perhaps the majority of strains of *botulinus* (Type A), since the order of frequency of fermentation of these sugars by different strains seems to be maltose, sucrose, and lactose (4).

Since the carbohydrate-splitting enzymes of *botulinus* bacilli had never been demonstrated, it seemed of interest to determine whether or not the sterile extract prepared from the intracellular substances of the bacilli possessed the property of hydrolyzing maltose. Tests were included with sucrose and lactose as representing disaccharides not fermented by the living cultures of this strain, as well as with maltose which is slowly fermented by the living culture.

Hydrolysis Mixtures.—Sterile solutions of maltose, sucrose, and lactose were prepared as follows: 30 per cent solutions of each of the sugars were boiled for 15 minutes and then added aseptically to sterile 0.1 M phosphate (pH 6.9) in amount sufficient to give a final concentration of 3.0 per cent of the sugar. The final solutions were distributed into sterile Pyrex tubes.

The hydrolysis test mixtures were prepared by adding 2.0 cc. of the *botulinus* enzyme solution to one tube of each of the three sugar solutions. (This relatively large amount of enzyme was used because preliminary experiments had shown that the maltase action of the *botulinus* extract was weak and difficult to demonstrate.) One portion of the enzyme solution was boiled for 15 minutes to inactivate the enzymes, and a series of controls of heat-inactivated enzyme was prepared for each of the substrates.

The mixtures of enzyme solution plus the sugar substrate were incubated for 72 hours and then tested for the presence of hexoses.

Detection of the Hexose Products of the Enzyme Action.—The hydrolysis of the disaccharides was tested by the biological method described in detail in preceding papers (3, 5). The method is based upon the acid fermentation of the hexoses by bacteria which do not attack the disaccharide from which the hexoses are derived. 1 cc. of each of the test and control hydrolysis mixtures was put into a sterile Pyrex test-tube; 1 cc. of a suspension of bacteria which produce acid from hexoses but which do not attack the test disaccharide was then added to the different tubes. (An atypical strain of colon bacilli which does not attack maltose was added to the

maltose tests; Shiga dysentery bacilli which do not attack maltose, lactose, or sucrose were used for the lactose and sucrose tests was well as for the maltose series.)

These "fermentation mixtures" (equal portions of hydrolysis test mixture plus the bacterial fermenting agent) were shaken, incubated in the water bath at 38°C. for 1 hour, and then centrifuged at high speed to remove the bacteria. The production of acid in the "fermentation mixtures" was detected by colorimetric estimations of the pH of the clear supernatant fluids of each of the test mixtures. The series of controls consisting of heat-inactivated enzyme plus the test disaccharide serve to limit all changes in reaction of the fermentation mixtures to acid produced by the fermentation of hexoses previously formed by the active *botulinus* enzyme.

The protocol of the experiment is summarized in Table I.

TABLE I.

Action of Botulinus Enzyme Solution upon Maltose.

Hydrolysis mixture		pH after action of bacteria used as fermenting agent	Change in pH due to bacterial fermentation of the hexose previously formed by *botulinus* enzyme
		pH	ΔpH[1]
Maltose	Active *botulinus* enzyme solution	7.0	0.6
	Heat-inactivated *botulinus* enzyme solution	7.6	0
Sucrose	Active *botulinus* enzyme solution	7.6	0
	Heat-inactivated *botulinus* enzyme solution	7.6	0
Lactose	Active *botulinus* enzyme solution	7.6	0
	Heat-inactivated *botulinus* enzyme solution	7.6	0

[1] ΔpH when used in Tables I to IV indicates change (decrease) in pH.

From the results of this experiment (Table I), it is evident that the *botulinus* enzyme solution contains an active maltase, which can be rendered inactive by heat. The maltose-splitting activity of the solution of the intracellular substances of the *botulinus* bacilli is relatively weak in comparison to the activity of the same type of bacterial solution prepared from either meningococci (3) or pneumococci (5). This relatively weak action of the sterile solution of the *botulinus* bacilli is paralleled by the slow fermentation of maltose by living cultures of the strain of *botulinus* from which the enzyme solution itself was prepared.

It is also evident in Table I that no acid is formed by the hexose-fermenting bacteria when added to previously incubated mixtures of the *botulinus* enzyme and lactose or sucrose solution. This fact may be accepted as proof of the absence of lactase or sucrase in the sterile extract of the *botulinus* bacilli. The failure of the sterile extract of the *botulinus* bacilli to hydrolyze either lactose or sucrose is in agreement with the inability of living cultures of the same strain to produce either acid or gas from the same disaccharides. Apparently,

TABLE II.

Splitting of Tributyrin by Sterile Extracts of Botulinus Bacilli.

Test mixture	Amount of bacterial extract	Reaction of test mixtures after 48 hrs. at 38°C.	Change in reaction due to splitting of tributyrin
	cc.	*pH*	*ΔpH*
Tributyrin plus active bacterial extract	0.8	6.3	1.2
	0.2	6.8	0.8
Tributyrin plus heat-inactivated bacterial extract	0.8	7.5	0
	0.2	7.6	0
Phosphate solution plus active bacterial extract	0.8	7.5	0
	0.2	7.6	0
Phosphate solution plus heat-inactivated bacterial extract	0.8	7.6	0
	0.2	7.7	0
Tributyrin solution plus broth	0.8	7.5	0
	0.2	7.6	0

the general rule (5) that the acid fermentation of disaccharides depends upon their preliminary hydrolysis to hexoses holds true for the anaerobic *botulinus* bacillus.

Demonstration of Lipase Action of Botulinus Enzyme Solutions.

The lipase action of the *botulinus* enzyme solution was demonstrated by tests of the production of acid from the splitting of tributyrin.

A 2 per cent solution of tributyrin prepared in 0.1 M phosphate (pH 7.8) was sterilized at 120°C. and 5.0 cc. portions of the sterile solution distributed into sterile tubes; 0.8 cc. and 0.2 cc. of the enzyme solution were then added to separate

tubes of the tributyrin. Three series of controls were included to limit the production of acid to that formed through the action of the enzyme on the tributyrin; (1) phosphate solution plus unheated enzyme; (2) phosphate solution plus heated enzyme; (3) tributyrin solution plus broth. The first two series served as controls on the possibility of changes in reaction due to changes in the enzyme solution itself; the third series controlled the possibility of a spontaneous hydrolysis of the tributyrin itself during the incubation period allowed for enzyme action. A fourth control series consisting of heat-inactivated enzyme plus tributyrin solution was included to prove that the lipase was a heat-labile substance.

The hydrolysis test mixtures and the series of controls were incubated at 38°C. for 48 hours. Tests for the splitting of the tributyrin were made by determining the changes in reaction of the various mixtures. The results are given in Table II.

The results presented in Table II furnish evidence of the action of lipase derived from *botulinus* bacilli. There is a direct relation between the extent of the hydrolysis and the concentration of the sterile bacterial extract.

Resistance of Botulinus Lipase to Exposure to Air.

The lipase of Pneumococcus is susceptible to oxidation and can be inactivated by treatment with an oxidizing agent, such as hydrogen peroxide (6), but it is considerably more resistant to oxidizing agents than are the carbohydrate-splitting enzymes of the same bacteria. Since the *botulinus* bacillus is an anaerobic organism, it seemed of particular interest to determine the effect of exposure to air upon the activity of its lipase enzyme.

The question of the effect of exposure to air was investigated from two points of view: (1) the effect of exposing the bacterial extract to air before its addition to the tributyrin solution; (2) the effect of free exposure of the mixture of tributyrin and bacterial extract during the incubation period allowed for the enzyme action.

The effect of previous aeration of the bacterial extract upon its subsequent lipase activity was studied as follows: 5.0 cc. of the sterile bacterial extract were placed in a shallow layer in a 300 cc. Erlenmeyer flask; another portion of the same bacterial extract was placed in a long, narrow test-tube, and carefully sealed with vaseline. Both the aerated and the sealed bacterial extract were held at 38°C. for 24 hours. Equal portions of the aerated and sealed extract were then tested for their lipase activity as described in the preceding experiment.

The effect of exposure of the hydrolysis mixture to air during the incubation period allowed for enzyme action was tested as follows: 4.0 cc. of tributyrin solution were placed in each of a series of narrow (11 mm.) test-tubes; the same amount of tributyrin solution was placed in a series of wide (50 mm.) tubes. 1.0 cc. and 0.3 cc. of the sterile bacterial extract (not previously exposed to air) were then added to separate narrow tubes of tributyrin solution; these tubes were then carefully sealed from air by a heavy layer of sterile vaseline. The same amounts of the same enzyme solution were added to the wide tubes of tributyrin; this series was not sealed with vaseline. The two series then represented hydrolysis mixtures, one of which was protected from air by a high column of fluid overlaid with vaseline, while the other was freely exposed to air in a shallow layer. Controls with heat-inactivated enzyme solution were included for both the sealed and aerated series.

TABLE III.

Effect of Exposure to Air upon the Lipase Activity of Sterile Extracts of Botulinus Bacilli.

Amount of bacterial extract	Effect of previous aeration upon subsequent lipase activity (enzyme solution aerated in absence of substrate)		Effect of aeration of the hydrolysis mixture during incubation period allowed for enzyme action (enzyme solution aerated in presence of substrate)	
	Change in reaction due to hydrolysis of tributyrin		Change in reaction due to hydrolysis of tributyrin	
	Bacterial extract previously exposed to air for 24 hrs.	Bacterial extract not previously exposed to air	Aerated hydrolysis mixture	Sealed hydrolysis mixture
cc.	ΔpH	ΔpH	ΔpH	ΔpH
1.0	1.5	1.5	1.4	1.4
0.3	0.8	0.8	0.8	0.8

After 60 hours incubation at 38°C., the degree of hydrolysis of the tributyrin was estimated by comparisons of the changes in reaction in the different test mixtures. The results are summarized in Table III.

As shown in Table III, previous aeration of the extract of the bacterial substances was without effect upon the subsequent activity of the lipase, and likewise the presence of air during the incubation of the enzyme-substrate mixture did not influence the action of the enzyme upon tributyrin. The resistance of the lipase to aeration is in striking contrast to the susceptibility of the living anaerobic cells from which the enzyme was derived.

Exocellular or Endocellular Nature of the Maltase and Lipase of the Botulinus Bacillus.

Experiments were carried out to determine whether the maltase and lipase of *botulinus* bacilli are endocellular or exocellular. These experiments consisted in comparisons of the activities of the supernatant fluid of a young unautolyzed broth culture with the activities of a sterile extract prepared by disrupture of the centrifuged bacterial cells of the same culture.

The preceding demonstrations of the lipase and maltase of *botulinus* were made with sterile extracts of the bacilli suspended in the supernatant fluids of broth cultures. Obviously, these enzyme solutions contained both the exocellular sub-

TABLE IV.

Exocellular or Endocellular Nature of the Maltase and Lipase of the Botulinus Bacillus.

Hydrolysis test mixture		
Source of enzymes	Substrate	
	Maltose	Tributyrin
Sterile bacterial preparation containing	Change in pH due to fermentation of glucose formed by *botulinus* maltase	Change in pH due to splitting of tributyrin by *botulinus* lipase
	ΔpH	ΔpH
Only exocellular products of *botulinus* bacilli	0.0	1.0
Both exocellular and endocellular products of *botulinus* bacilli	0.5	1.0

stances liberated during the growth of the culture and the intracellular substances set free by the disintegration of the suspended bacteria. For the following experiment, it was necessary to obtain a sterile solution containing only the exocellular products which are liberated into the culture fluid during the period of active growth and before bacterial dissolution had progressed.

1. Solution Containing Only Exocellular Substances.—The fluid used as the solution of exocellular substances consisted of the supernatant fluid obtained by centrifugation of an 18 hour broth culture.

2. Solution Containing Both Exocellular and Endocellular Substances.—The bacterial solution was prepared from the sedimented bacterial cells of the same culture as that used for Solution 1. The bacilli were suspended in a small amount of the supernatant fluid as described under Methods for the preparation of bacterial extracts, and subjected to the freezing and thawing process.

Both the supernatant fluid and the bacterial solution were finally filtered

through a Berkefeld filter. The comparison of the maltase and lipase activities of the two preparations was made by the procedure used in the preceding experiments. The results are summarized in Table IV.

The results of this experiment (Table IV) show a difference in the nature of the maltase and lipase of *botulinus*. The maltase must be endocellular in nature, since maltose is hydrolyzed only by the sterile preparation which contains the intracellular substances of the bacilli and not by the sterile filtrate which contains only the *botulinus* products of exocellular origin. The lipase, on the other hand, seems to represent an extracellular rather than an intracellular substance, since tributyrin is split as actively by the sterile *botulinus* filtrate which contains only exocellular products as by the extract of the bacterial substances. The agreement in the extent of glyceride hydrolysis by the two *botulinus* preparations indicates that no lipase is contained within the *botulinus* bacilli themselves. Thus, while the endocellular maltase can be accepted as an actual constituent of the *botulinus* bacterial cell, the glyceride-hydrolyzing enzyme must be considered a substance of different nature which the bacillus liberates into the culture fluid during the period of active growth before bacterial autolysis occurs.

DISCUSSION.

This investigation presents experimental evidence of the maltase and lipase of the *botulinus* bacillus and thus demonstrates the enzymatic nature of two biochemical activities of this anaerobic organism. The proof of the maltase not only shows that *botulinus* bacilli possess an enzyme capable of hydrolyzing maltose in the absence of the bacteria themselves, but furnishes evidence that the capacity of these anaerobic bacilli to ferment different disaccharides depends upon their possession of a specific enzyme to hydrolyze the disaccharide to its component hexoses. The sterile bacterial extract prepared from a strain of *botulinus* which ferments maltose, but not lactose and sucrose, contains an active maltase but no lactase or sucrase.

The maltase enzyme, like the carbohydrate-splitting enzymes of certain aerobic bacteria, as pneumococci (5–7) and meningococci (3), is endocellular in nature, and hence is an actual constituent of the *botulinus* bacterial cell. The lipase, on the other hand, is an extracellular substance, and is extruded into the culture fluid during the period of growth of the bacteria. The lipase of *botulinus* differs in this respect from the endocellular one of Pneumococcus (6, 7) but agrees with those from a number of other aerobic bacteria (8) which also are exocellular in nature.

The results of the experiments presented show that the lipase of *botulinus* is resistant to exposure to air. It was found that 24 hours

aeration of the bacterial extract at 38°C. had no detectable effect upon the subsequent activity of this enzyme; similarly, free exposure to air of the enzyme-substrate mixtures during the incubation period allowed for enzyme action did not seem to affect its activity. The indifference to air which is shown by the heat-labile lipase of *botulinus* is in striking contrast to the susceptibility of the living bacillus. If oxygen be considered toxic to anaerobes, the resistance to oxidation shown by the lipase of these anaerobic bacilli is analogous in some respects to other known examples of the relative resistance of individual cell products to deleterious influences which are toxic to the living cell as a whole. Frequent examples are known of enzymes of aerobic bacteria which withstand heating treatment which kills the cell itself; similarly, concentrations of disinfectants which promptly end the life of the bacterial cell as a whole, often have but a slight inhibitory effect upon many of the enzymes derived from the same bacteria. Nevertheless, in spite of the frequency with which somewhat similar phenomena are encountered, it is of considerable interest to observe that the fat-hydrolyzing enzyme of this anaerobic bacillus, after its elaboration, is apparently indifferent to conditions of oxygen tension which render impossible the growth of the bacilli from which the enzyme is derived.

SUMMARY.

Botulinus bacilli yield a maltase and a lipase, which retain their hydrolytic activity independent of the presence of the formed bacterial cell. The maltase is an endocellular substance, while the lipase is extracellular. The lipase, when separated from the anaerobic bacillus, does not seem to be affected by exposure to air.

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STUDIES ON BACTERIAL ENZYMES.

V. The Carbohydrases and Lipase of the Welch Bacillus.

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(Received for publication, March 4, 1927.)

INTRODUCTION.

Gas production is the most prominent metabolic process of *B. aerogenes capsulatus*—the Welch bacillus—both in test-tube culture and in the animal body. It is desirable, therefore, to obtain further knowledge of the reactions involved in the fermentation of higher carbohydrates by this anaerobic organism. Previous studies on pneumococci (1–3) and meningococci (4) have demonstrated carbohydrate-hydrolyzing enzymes and also their hexose products, and thus constitute proof that hydrolysis represents a preliminary reaction in the fermentation of complex sugars by these particular species of bacteria. However, the products formed in the Welch bacillus fermentations are quite different from those formed from the same sugars by the bacteria with which the hydrolyzing enzymes have actually been demonstrated. This fact, together with the reports in the literature (5) of polysaccharide fermentation by other bacteria without preliminary hydrolysis, makes it desirable to seek experimental proof of the carbohydrases of the Welch bacillus rather than to assume their presence from observations that the specific carbohydrate is fermented by the living bacilli. Conversely, the demonstration of the enzymes themselves and of their hexose products furnishes at the same time indirect evidence that the higher carbohydrate is not attacked without preliminary hydrolysis.

This investigation also deals with the effect of oxidation upon the activity of the Welch bacillus enzymes. The deleterious influence of air upon the growth of strict anaerobic bacteria makes it evident

that oxygen must have a toxic or inhibitory effect upon at least some of the cellular agents involved in life processes of the bacterial cell. While it seems probable that different cellular constituents vary in their susceptibility to air, so many different agents are concerned in the complex functions and processes of "growth" and "life" that it is impossible to determine, with living cultures, the relative sensitivity of the individual components. With "enzyme solutions" or sterile extracts of the bacterial bodies, the various cellular components have already been formed under optimum conditions for growth, and hence can subsequently be tested in respect to their individual susceptibility to oxidation without introducing all of the complications entailed in the "growth" of living cultures of the bacilli.

EXPERIMENTAL.

Methods.

Preparation of the Sterile Bacterial Extracts or Enzyme Solutions.—The sterile extracts or enzyme solutions were prepared from a typical strain of the Welch bacillus by a method essentially the same as that previously described for the preparation of sterile extracts of *botulinus* bacilli (6). The Welch bacilli offer considerable resistance to disintegration by freezing and thawing and it was necessary to repeat the process 150 times to obtain satisfactory extracts. After Berkefeld filtration, the bacterial extract was perfectly clear, but contained a considerable amount of dissolved bacterial substance as indicated by the marked precipitation of coagulated protein when boiled.

Sterility Controls.—No antiseptics were used; the sterility of each enzyme-substrate mixture was controlled by cultural methods.

Substrates.—The solutions of carbohydrates and tributyrin were prepared in the same manner as described in the preceding paper. Unless otherwise specified, air was excluded from all of the hydrolysis test mixtures during the incubation period allowed for enzyme action by preparing them in long, narrow tubes sealed with a heavy layer of vaseline.

Carbohydrate Hydrolysis by Enzymes Contained in Sterile Extracts of Welch Bacilli.

5 cc. portions of sterile, 3 per cent solutions of maltose, lactose, sucrose, raffinose, and starch were distributed into five series of sterile Pyrex test-tubes. 0.2 cc. of the sterile bacterial extract was added to one tube of each of the substrates. A second series was then prepared to serve as controls of the heat lability of the enzymes; the same amount of heat-inactivated bacterial extract was

added to each of these tubes. All of the mixtures were then sealed with vaseline to exclude air, and incubated at 38°C. for 48 hours.

At the end of this incubation period, tests were made for the presence of the products of enzyme action. The sucrose, raffinose, and starch series were tested with Benedict's solution, and the approximate per cent of reducing sugars estimated by comparison with standard glucose solutions. With the lactose and maltose hydrolysis test mixtures (where the substrates themselves are reducing sugars) it was more convenient to detect the hexoses by the biological method described in previous papers (4, 3, 6).

Living cultures of Welch bacilli actively attack the hexoses which would be formed by enzymes hydrolyzing the above substrates. Hence, tests were made

TABLE I.

Splitting of Carbohydrates by Enzymes Contained in Sterile Extracts of Welch Bacilli.

Substrate	Carbohydrate hydrolysis		Evidence of action on hexose products	
	Biological tests for presence of hexose products of enzyme action	Chemical tests for reducing sugars		
	Change in pH due to hexoses previously formed by Welch bacillus enzymes	Per cent reducing sugars	Acid production	Gas production
	ΔpH[1]	*per cent*		
Maltose	1.5	Not tested	0	0
Lactose	1.2	Not tested	0	0
Sucrose	Not tested	0.8	0	0
Raffinose	Not tested	0.6	0	0
Starch	Not tested	0.3	0	0

[1] ΔpH when used in Tables I to IV indicates change (decrease) in pH.

to see whether or not the sterile extract of the Welch bacilli produced either acid or gas in the hydrolysis mixtures in which the hexoses had accumulated during the period of enzyme action.

The results are summarized in Table I.

The results of these experiments (Table I) show that sterile extracts of the bacterial substances of Welch bacilli contain enzymes which actively hydrolyze maltose, lactose, sucrose, raffinose, and starch. Living cultures of Welch bacilli attack all of these complex carbohydrates with the vigorous production of acid and gas. In contrast to the living bacteria the sterile extracts are without action upon the

hydrolytic products of the above carbohydrates, as evidenced by their failure to form either acid or gas in mixtures containing a relatively high concentration of the fermentable hexoses. Other tests were made with 1 per cent glucose both in phosphate solution and in broth, and in these tests the bacterial extract was likewise without action upon the hexose which yields both acid and gas in the presence of the living Welch bacillus.

The logical explanation of these phenomena is the same as that previously made for similar results with sterile extracts of pneumococci (3) and meningococci (4). The living Welch bacillus attacks complex carbohydrates through a preliminary splitting to hexoses by a specific hydrolytic enzyme, and then subsequently ferments the hexose by means of other cellular processes. Although these two reactions are probably simultaneous in the living cell, they represent two distinct and separate processes, since only one of them (the hydrolytic property) retains its activity in sterile filtered solutions of the intracellular substances (7). Thus, the above experiment furnishes experimental evidence of the order of reactions operative in the fermentation of complex carbohydrates by Welch bacilli.

The ability of different strains to attack a specific polysaccharide depends upon their possession of the requisite hydrolyzing enzyme. Living cultures of the strain from which the above bacterial extract was derived, vigorously fermented all of the substrates listed in Table II. The same strain is without action upon inulin and similarly the bacterial extract seems to be totally devoid of an inulin-hydrolyzing enzyme.

Splitting of Tributyrin by Sterile Extracts of Welch Bacilli.

While the present paper is concerned principally with carbohydrate-hydrolyzing enzymes, the sterile extracts of Welch bacilli also contain an active enzyme which hydrolyzes tributyrin. Experimental evidence of the splitting of the glyceride by a heat-labile agent derived from the Welch bacillus is presented in the protocol given in Table II.

As shown by these results (Table II), the heat-labile lipase of this anaerobic bacillus retains its ability to hydrolyze tributyrin in the complete absence of living or formed bacterial cells. It is interesting to observe that the Welch bacillus apparently possesses a much less

active lipase than does the *botulinus* bacillus. Comparison with the results given in the preceding paper (6) makes it evident that much larger amounts of the Welch bacillus extracts are required to cause the same degree of hydrolysis than in the case of the enzyme solution

TABLE II.

Splitting of Tributyrin by Sterile Extracts of Welch Bacilli.

Hydrolysis test mixture	Amount of bacterial extract	Reaction of test mixtures after 48 hrs. incubation at 38°C.	Change in reaction due to splitting of tributyrin by enzyme action
	cc.	*pH*	*ΔpH*
Tributyrin plus active bacterial extract	3.0	6.5	0.9
	0.5	7.2	0.4
Tributyrin plus heat-inactivated bacterial extract	3.0	7.4	0
	0.5	7.6	0

TABLE III.

Endocellular Nature of Carbohydrases and Lipase of the Welch Bacillus.

Sterile bacterial preparation	Substrates				Enzyme activity
	Maltose	Sucrose	Starch	Tributyrin	
	Change in pH due to fermentation of glucose formed by Welch enzymes	Reducing sugars by Benedict test	Reducing sugars by Benedict test	Change in pH due to splitting of the glyceride	
	ΔpH	*per cent*	*per cent*	*ΔpH*	
Bacterial extract (filtered solution of intracellular substances)	1.2	0.8	0.3	0.8	+
Filtrate of supernatant of young broth culture (containing only exocellular substances)	0.0	0.0	0.0	0.0	0

derived from *botulinus*. The maltase action of the Welch bacillus preparation, on the other hand, is much more pronounced than was that of the bacterial extract prepared from the *botulinus* bacilli, a relation which is paralleled by the metabolic activities of living cultures of the same bacteria.

Endocellular Nature of the Carbohydrases and Lipase of Welch Bacilli.

The test of the endocellular or exocellular nature of the enzymes consisted in a comparison of the enzyme activity of the bacterial extract containing both intracellular and extracellular products with the activity of the culture filtrate which contained only extracellular substances.

The protocol is summarized in Table III.

It is evident (Table III) that the carbohydrases and the lipase of the Welch bacillus are endocellular in nature. Its carbohydrases agree in this respect with the similar enzymes of Pneumococcus (1–3, 8, 10), Meningococcus (4, 9), and the *botulinus* bacillus (6). The Welch bacillus lipase agrees with that of Pneumococcus (8, 10) while it differs from the lipase of *botulinus* which is an extracellular substance not retained within the bacterial cell itself but liberated into the environment during the period of active growth of the bacillus (6).

Our real interest in the endocellular nature of these enzymes of the Welch bacillus is to furnish a basis for following experiments on their oxidation and inactivation by exposure to air. These enzymes have been proved in the above experiment to be endocellular in nature and not liberated into the culture fluid until the bacterial cells are disintegrated, and consequently, it is necessary to accept them as actual components of the bacterial cell.

Relative Resistance to Oxidation Shown by the Carbohydrases and the Lipase of the Welch Bacillus.

Tests were made of the effect of previous aeration of the bacterial extract upon the subsequent activity of enzymes; tests were also made of the inactivating effect of treatment with different concentrations of hydrogen peroxide, a chemical oxidizing agent which is identical or similar to those involved in many biological oxidations.

One portion of the sterile bacterial extract was freely exposed to air for 30 hours at 38°C. in a shallow layer in an Erlenmeyer flask. Hydrogen peroxide ("dioxogen" diluted in sterile pH 7.5 phosphate solution) was added to a second portion of the same extract in amount sufficient to give a concentration of 10 mM H_2O_2; the same reagent was added to a third portion of the extract to give a concentration of 100 mM H_2O_2; the H_2O_2-treated extract was also stored at 38°C. for 30 hours in long narrow tubes sealed with vaseline. Another portion of the original extract was stored under similar conditions to serve as a control of the

activity of the unoxidized or unaerated extract. A fifth portion of extract was boiled for 10 minutes as a control for all of the hydrolysis mixtures.

For the subsequent tests of maltase and lipase activity, the following hydrolysis mixtures were prepared: (1) substrate plus enzyme solution which had been protected from air; (2) substrate plus the enzyme solution which had previously been exposed to air for 30 hours; (3) substrate plus the enzyme solution exposed to 10 mM H_2O_2; (4) substrate plus the enzyme solution exposed to 100 mM H_2O_2; (5) substrate plus enzyme inactivated by heat (boiled).

The tests of the enzymes attacking sucrose, raffinose, and starch included only three of the above series, and consisted of the following mixtures: (1) substrate plus enzyme solution which had been protected from oxidation; (2) substrate plus enzyme exposed to 100 mM H_2O_2; (3) substrate plus heat-inactivated enzyme solution.

TABLE IV.

Relative Resistance to Oxidation Shown by the Carbohydrases and Lipase of the Welch Bacillus.

Treatment of bacterial extract	Lipase	Maltase	Sucrase	Raffinase	Amylase
	Change in pH due to splitting of tributyrin by Welch bacillus enzyme	Change in pH due to fermentation of glucose formed by Welch bacillus enzyme	Reducing sugars	Reducing sugars	Reducing sugars
	ΔpH	*ΔpH*	*per cent*	*per cent*	*per cent*
Bacterial extract stored at 38°C. but protected from air	0.8	1.5	0.9	0.7	0.4
Exposed to air for 30 hrs. at 38°C.	0.9	1.0	Not tested	Not tested	Not tested
Exposed to 10 mM H_2O_2	0.8	0.6	Not tested	Not tested	Not tested
Exposed to 100 mM H_2O_2	0.4	0.0	0.0	0.0	0.0
Boiled for 10 min.	0.0	0.0	0.0	0.0	0.0

All of the above hydrolysis mixtures were prepared in narrow Pyrex tubes and were sealed with vaseline to exclude air during the incubation period allowed for enzyme action. After 72 hours incubation at 38°C., the test mixtures were examined by the methods used in preceding experiments.

The results of the experiment are summarized in Table IV.

As shown in Table IV, the maltase suffered a significant loss in subsequent activity when the bacterial extract was exposed to air for

30 hours, or when it was exposed to a relatively low concentration of H_2O_2 (10 mM, or approximately 0.03 per cent). The lipase, on the other hand, did not seem to be affected at all by either of these two oxidation treatments. In the tests with a higher concentration of H_2O_2 (100 mM), similar relations were evident: while exposure to this oxidation treatment inactivated completely the carbohydrases (maltase, sucrase, raffinase, amylase), the same conditions caused only a partial inactivation of the lipase. These facts are evidence that the lipase of the Welch bacillus is much more resistant to oxidation than are its carbohydrases, whether the oxidative processes are induced by aeration or by treatment with hydrogen peroxide.

Since the carbohydrases and lipase of the Welch bacillus must be accepted as actual constituents of the cell, the above results acquire interest as examples of differences in the relative sensitivity to oxidation which are possessed by the various cellular components of anaerobic bacilli. The greater susceptibility to oxidation is apparently a characteristic of bacterial carbohydrases. The carbohydrate-splitting enzymes of Pneumococcus are much more easily inactivated by oxidation than are their glyceride-splitting and peptone-splitting enzymes (2, 3, 10); similarly, the maltase of meningococci is much more readily oxidized than is the peptonase of the same bacteria (9).

Influence of Aeration of the Enzyme-Substrate Mixture during the Incubation Period Allowed for Enzyme Action.

The preceding experiment dealt with the effect of previous aeration of the bacterial extract itself upon the subsequent activity of the enzyme. The object of the present experiment is to determine whether or not free exposure of the enzyme-substrate mixture to air during the period of enzyme action affects the activity of the same enzymes.

The experiment was similar to that previously made in the investigation of the same question with *botulinus* lipase (6). Two series of both tributyrin-enzyme mixtures and of maltose-enzyme mixtures were prepared. One series was incubated in a narrow tube sealed from air with a heavy layer of vaseline. The second series was exposed to air by placing the enzyme-substrate mixture in a wide tube; this fluid was subjected to free aeration as a wide surface area was exposed and the layer of fluid was shallow (approximately 2 mm.).

Both the aerated series and the series protected from air were incubated for 48

hours at 38°C. The extent of enzyme action was then determined by the methods employed in previous experiments.

The results of the above experiments were conclusive. The lipase, as in the previous study of the similar enzyme of *botulinus*, was indifferent to the presence of air and exhibited the same degree of activity in the aerated hydrolysis mixture as in the mixture protected from air. Even the labile maltase was not affected to a detectable degree by the presence of air in the enzyme-substrate mixture. Although the maltase was inactivated to a significant extent in the previous experiment where the undiluted bacterial extract was aerated before its addition to the substrate, the active enzyme itself hydrolyzed maltose to the same extent when the enzyme-substrate mixture was exposed to air as when air was excluded during the period of enzyme action.

While the maltase appears to be relatively indifferent to aeration of the enzyme-substrate mixtures during the period of enzyme action, it is partially inactivated when the bacterial extract itself is exposed to air previous to the preparation of enzyme-substrate mixtures. Sufficient data are not at hand to explain this apparent difference in the resistance of the maltase to the two conditions of aeration. Other workers (11) have reported similar differences in the destruction of enzymes, where the enzyme inactivation has been less pronounced if the substrate is present during the exposure of the enzyme to a deleterious influence than if the enzyme is subjected to the same conditions in the absence of the substrate. While the above phenomenon in the case of Welch bacillus maltase may also be due at least in part to a similar "protective action of the substrate," we believe that an important factor in our experiment is the difference in the dilution of the bacterial extract under the two sets of conditions. In the first instance, air was allowed to act upon the undiluted bacterial extract; in the second experiment, where the enzyme-substrate mixture was exposed to air, the bacterial substances were present in only about one-tenth their original concentration. If the inactivation of the maltase through oxidation by air is not a first order reaction, the dilution itself would decrease the speed of the inactivating processes.

DISCUSSION.

The hydrolysis of tributyrin and of a number of different carbohydrates by heat-labile agents present in sterile filtered solutions of the intracellular substances of the Welch bacillus proves that the action of its lipase (tributyrinase) and carbohydrases (maltase, lactase,

sucrase, raffinase, amylase) is independent of the presence of either the living or formed bacilli. The further study of these enzymes can contribute to our knowledge of the mechanism of metabolism and method of growth of this pathogenic organism.

The results of the experiments on the carbohydrases show that the fermentation of complex carbohydrates, a characteristic metabolic process of the living Welch bacillus, is dependent upon the presence of specific hydrolyzing enzymes. Since the sterile bacterial extract which contains the active hydrolases is devoid of any action upon the hexose products, it is possible to detect intermediate products which would be rapidly consumed in experiments with the living bacilli. Thus, the above demonstration of the carbohydrate-hydrolyzing enzymes of the Welch bacillus constitutes experimental evidence that Welch bacilli attack complex carbohydrates by way of two separate sets of reactions: (1) hydrolysis; (2) fermentation of the hexose products of the hydrolytic reaction. Of the two reactions (hydrolysis and fermentation) which are effected by the living cell, only the hydrolytic property retains its activity in sterile, filtered solutions of the bacterial substances.

Exposure of the bacterial extract to air resulted in no detectable loss in the subsequent activity of the lipase, but caused a slight loss in the activity of the more labile carbohydrases. Treatment of the bacterial extract with a low concentration of hydrogen peroxide, although entirely without effect upon the lipase, caused a partial inactivation of the maltase; a higher concentration of peroxide which was sufficient to induce only a partial inactivation of the lipase caused the complete inactivation of all of the carbohydrases. Thus, although both the lipase and the various carbohydrases are affected by sufficiently strong oxidation treatment, the lipase is more resistant than the carbohydrases whether the oxidative processes be induced by air or by hydrogen peroxide. The lipase and carbohydrases are intracellular substances and hence must be considered as integral constituents of the Welch bacillus cell. From this point of view, the greater sensitivity of the Welch bacillus carbohydrases in comparison to the lipase presents the first real demonstration of the differences in susceptibility to oxidative inactivation which might be expected to exist between individual cellular components of anaerobic bacteria.

Although exposure of the anaerobic extract to air previous to its addition to the substrate caused a slight but definite inactivation of the carbohydrases, air had no effect when the exposure was made in the presence of the substrate during the period of enzyme action. In fact, so far as we could determine, all of the enzymes (the more labile carbohydrases as well as the lipase) hydrolyzed their substrates as effectively in the presence of air as in its absence. The indifference of the enzymes to air under these conditions is in striking contrast to the extreme susceptibility of the living cell from which the enzymes were derived. The fact that the presence of air inhibits growth of strict anaerobic bacteria makes it evident that oxygen must have a toxic or inhibitory effect upon at least some of the cellular agents involved in certain life processes of the anaerobic cell. Since the various components of a cell differ in their individual resistance toward deleterious agents, the inhibition of growth of the cell as a whole would be determined by the specific sensitivity of the most labile component, if this be involved in a process inherently essential to the life of the organism. One would be inclined to limit the category of indispensable life processes to the energy-productive reactions concerned in the initiation of growth of the cells. Since the hydrolyzing enzymes are not directly concerned in reactions of this type, it is extremely unlikely that their functions would ultimately determine the growth of the culture in any environment. However, in spite of these relations, it is of interest to obtain experimental evidence that the inhibitory effect of air upon the initiation of growth of the Welch bacillus does not include a comparable effect upon the activity of its hydrolyzing enzymes. In fact, the evidence indicates that there is no essential difference between the hydrolytic enzymes of aerobic and anaerobic bacteria in respect to sensitivity to oxidation.

The slight degree of inactivation of the carbohydrases when an extract of Welch bacilli is exposed to air is by no means comparable to the rapid and almost complete inactivation of carbohydrases which occurs under the same conditions in a similarly prepared extract of pneumococci. The apparent difference in the effect of air upon the carbohydrases contained in extracts of pneumococci and of Welch bacilli is not in itself important, but it is desirable to analyze the factors involved since the same fundamental principles are concerned in

the oxidation of many other biological substances. When oxidative inactivations take place in complex biological systems, an explanation of apparent differences in the resistance of substances in these systems (12) requires the consideration of at least two factors: (1) the intrinsic susceptibility to oxidation of the substances themselves; (2) the oxidizing activity of the systems in which the substances are exposed to air.

In the present instance, there is no evidence to support the possibility of differences in the intrinsic susceptibility to oxidation of the carbohydrases derived from the two bacteria, but there is convincing evidence that the two *systems* in which the carbohydrases are contained differ materially in oxidizing activity. First, peroxides are formed in significant and easily detectable concentrations in aerated pneumococcus extracts (13), while similar oxidizing agents, if produced in the Welch bacillus extract, do not accumulate in detectable amounts. Second, when a known substance, hemoglobin, is added to the two systems, it is likewise oxidized much more rapidly and more completely in the system (pneumococcus extract (7, 14)) in which the oxidation of the enzyme is more pronounced. Finally, when the system in which the pneumococcus enzymes are contained is changed to one in which peroxides are not formed (by preparing an extract from washed cells suspended in salt or phosphate solution (2)) the pneumococcus carbohydrases are fully as resistant to air as are those of the Welch bacillus. The above analysis is of interest as a definite illustration of the importance of the oxidizing activity of the *system* in determining the resistances of different biological substances to air; the same factor is probably involved in other instances where the oxidizing activity of the complex system is not so easily recognized.

SUMMARY.

The carbohydrases (maltase, lactase, sucrase, raffinase, amylase) and lipase (tributyrinase) of the Welch bacillus retain their hydrolytic activities in sterile solutions of the bacterial cells. The demonstration of the carbohydrases and detection of their hexose products constitute experimental proof that Welch bacilli attack complex carbohydrates by way of a preliminary hydrolysis.

The results of experiments on the oxidation of the enzymes show that the inhibitory effect of air upon the initiation of growth of the living Welch bacillus does not include a comparable effect upon the activity of its hydrolyzing enzymes.

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VARICELLA IN MONKEYS.

Nuclear Inclusions Produced by Varicella Virus in the Testicles of Monkeys.

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The lesions observed in testicles of monkeys inoculated with emulsified human varicella papules and vesicles have been described in a previous paper (1). In the experiments reported at that time several species of monkeys were employed and also rats, rabbits, guinea pigs, and chickens. Significant lesions, nuclear inclusions, were found only in the testicles of African vervets 5 and 6 days after inoculation, and not in other inoculated tissues of the same animals, nor in the other experimental animals. Furthermore, similar inclusions were not found in the testicles of a vervet inoculated with normal skin. The inclusions were the eosin-staining nuclear bodies which are consistently associated with certain virus diseases and which, regardless of their nature, indicate to many workers the presence of a virus. Therefore, in view of these facts, it was deemed not unlikely that the acidophilic nuclear inclusions in the vervets' testicles were manifestations of the presence of a virus. The nature of the virus had not been studied at the time of the previous report. Recently, however, experiments were performed to obtain, if possible, additional information concerning the suspected virus and it is with the results of this work that the present paper deals.

Methods and Materials.

Monkeys Employed.—It was impossible to obtain vervets (*Cercopithecus lalandi*). A search for susceptible animals was made among other *Cercopithecus* monkeys. A few experiments showed that green monkeys (*Cercopithecus sabæus*) very closely allied to vervets were satisfactory for the work. It is essential that the monkeys be young, and all animals in which spermatogenesis had been established were discarded.

Inoculations.—Emulsified papules and vesicles collected from varicella patients, usually within the first 72 hours of the disease, were used as virus. The papules and vesicles were excised under aseptic conditions and emulsified by grinding in a mortar moistened with Locke's solution. Sand was not used. The emulsified material was taken up in 0.5–1.0 cc. of Locke's solution and portions of it were mixed as desired with equal amounts of Locke's solution, non-immune serum, or immune serum. The mixtures were then injected into monkeys. More than 45–60 minutes never elapsed between the collection of the virus from the patients and its inoculation into animals. All monkeys were inoculated intratesticularly (0.2–2.5 cc.). The non-immune serum was obtained from varicella patients usually within the first 72 hours of the disease. The immune serum was obtained from convalescent varicella patients 14–22 days after the onset of the disease.

Removal and Examination of Testicles.—In previous experiments (1) it was determined that nuclear inclusions were present in the testicles on the 5th and 6th days after inoculation. Therefore in the experiments reported at the present time the monkeys were castrated* on the 5th day. Testicles removed for histological studies were fixed in Zenker's fluid with 5 per cent acetic acid, sectioned, and stained with eosin and methylene blue. A careful search for eosin-staining nuclear inclusions was made in numerous sections of each testicle. Details concerning the tinctorial reactions of the inclusions are given by Tyzzer (2), Lipschütz (3), Goodpasture (4), and others.

EXPERIMENTAL.

The experiments to be reported were conducted to determine whether the eosin-staining nuclear inclusions in monkeys' testicles inoculated with emulsified varicella papules and vesicles are specifically associated with the virus of varicella. In connection with this phase of the work six experiments, consisting of neutralization and reinoculation tests, were performed, and a detailed account of each is given below.

Experiment 1.—Monkeys L and M; green. December 23, 1925. 2 lesions were removed from each of 3 varicella patients, Cases 18, 19, and 20, 3 days after the onset of the disease. The papules and vesicles were emulsified together and taken up in 0.5 cc. of Locke's solution. The emulsion was divided into equal portions. Case 18 was bled on the 3rd day of the disease for non-immune serum; Case 17 was bled for immune serum 17 days after onset of the disease. Equal amounts of the sera, not inactivated, were mixed respectively with the two portions of emulsified papules and vesicles. The mixtures were injected immediately

* All operative procedures were conducted under ether anesthesia.

into 2 green monkeys as follows: Monkey L, 0.25 cc. of virus and immune serum in each testicle; Monkey M, 0.25 cc. of virus and non-immune serum in each testicle. The 4 testicles, removed 5 days later, were fixed and examined in the usual way for the presence of eosin-staining nuclear inclusions.

Although nuclear inclusions were not found in the testicles of Monkey L inoculated with virus and immune serum, typical ones were observed in the testicles of Monkey M inoculated with virus and non-immune serum.

Experiment 2.—Monkeys N and O; green. January 4, 1926. 5 lesions, vesicles and papules, removed from a varicella patient, Case 22, 48 hours after the appearance of the rash were emulsified and taken up in 0.5 cc. of Locke's solution. The emulsion was divided into equal portions. Non-immune serum was also obtained from Case 22 and was inactivated at 56°C. for 30 minutes. Convalescent serum from Case 17, collected and inactivated, December 23, 1925, was used as the immune serum. Equal amounts of the sera were mixed respectively with the two portions of virus emulsion. 2 green monkeys were inoculated immediately; Monkey N, 0.25 cc. of virus and immune serum in each testicle; Monkey O, 0.25 cc. of virus and non-immune serum in each testicle. Monkey N also received 3 cc. of the convalescent serum intraperitoneally; Monkey O, 3 cc. of the non-immune serum in a similar manner. 5 days later the monkeys were castrated. The testicles were fixed, sectioned, and examined in the usual way for the presence of nuclear inclusions.

Nuclear inclusions were not observed in the testicles of Monkeys N and O. No explanation of their absence from the testicles of Monkey O has been found.

Experiment 3.—Monkeys P and Q; green. January 8, 1926. 5 lesions, vesicles and papules, were removed from varicella patients,—2 from Case 24 on the 4th day of the disease, 3 from Case 25 within 36 hours after the appearance of the rash,—and ground up together. The emulsified material was taken up in 0.5 cc. of Locke's solution and divided into equal portions. The inactivated non-immune serum was a mixture of sera collected from Cases 18, 22, 24, and 25 on the 3rd, 2nd, 4th, and 2nd days of the disease respectively. The inactivated immune serum was a mixture of sera collected from Cases 17, 18, 21, and 23 on the 17th, 16th, 14th, and 14th days respectively after the appearance of the rash. Equal amounts of the non-immune and immune sera were mixed respectively with the two portions of virus emulsion. The mixtures were injected into 2 green monkeys as follows: Monkey P, 0.25 cc. of virus and immune serum in each testicle; Monkey Q, 0.25 cc. of virus and non-immune serum in each testicle. Monkey P also received 7 cc. of the immune serum intraperitoneally; Monkey Q,

7 cc. of the non-immune serum in a similar manner. 5 days later the testicles were removed, fixed, and examined in the usual way for the presence of nuclear inclusions.

Eosin-staining nuclear inclusions were found in the sections from Monkey Q inoculated with virus and non-immune serum. None were seen, however, in the sections from Monkey P inoculated with virus and immune serum.

Experiment 4.—Monkey A (1); vervet. November 12, 1924. 10 cc. of blood collected from a varicella patient, Case 1, 36 hours after the appearance of the eruption, was injected intravenously into Monkey A. Fluid from 30 vesicles was also collected at the same time and injected intradermally in left eyelid, in left and right thighs, and in right side of abdominal wall. While under observation the animal showed no manifestations suggestive of chicken-pox. No tissue was removed at this time for histological study.

Monkey A (1) was inoculated again, April 7, 1925. Emulsified varicella vesicles and papules from Cases 13 and 14 were injected into the right testicle. 6 days later the testicle was removed and fixed. Nuclear inclusions were not found in the sections.

Monkey A; Monkey R, green. January 9, 1926. 3 lesions were removed from varicella patients,—2 from Case 26 on the 2nd day of the disease, 1 from Case 27 on the 4th day of the disease,—emulsified, taken up in 0.5 cc. of Locke's solution. 0.25 cc. of the emulsion was injected into the left testicle of each monkey. Monkey R also received 7 cc. of fresh unclotted blood from Case 26. 5 days later the inoculated testicles were removed and fixed. A search was made in the usual manner for the presence of nuclear inclusions.

Monkey A was considered to be immune because of the two previous inoculations. Monkey R was normal and approximately as old as Monkey A. Nuclear inclusions were found in the normal testicle inoculated with varicella virus. None were seen, however, in the immune testicle inoculated with the same virus.

Experiment 5.—Monkey R, green. January 9, 1926. Monkey R was employed in Experiment 4. At that time the left testicle was inoculated with varicella virus. It was removed 5 days after the inoculation and sections of it showed nuclear inclusions.

Monkeys R, S, and T; green. February 3, 1926. 2 papules and 2 vesicles were removed from a varicella patient, Case 28, within 48 hours after the appearance of the rash, emulsified, and taken up in Locke's solution. There was 0.75 cc. of emulsion. The non-immune serum was collected from Case 25 on the 2nd day of the disease and inactivated. The immune serum was collected from

the same patient 12 days later and inactivated. 0.25 cc. of the virus emulsion was inoculated in the right testicle of Monkey R. 0.25 cc. of the emulsion was mixed with 0.25 cc. of the non-immune serum and then half of the mixture was injected into each testicle of Monkey S. 0.25 cc. of the virus emulsion was mixed with an equal amount of immune serum and then half of the mixture was injected into each testicle of Monkey T. In addition to the above injections Monkeys S and T received intraperitoneally 3.5 cc. of the non-immune and immune sera respectively. The testicles were removed and fixed 5 days later. A careful search through numerous sections revealed the presence of nuclear inclusions in both testicles of Monkey S. None were seen, however, in the testicles of Monkeys R and T.

Experiment 5, in which reinoculation and neutralization tests were conducted simultaneously, is especially interesting. Monkey R's left testicle inoculated with varicella virus, January 9, 1926, showed nuclear inclusions 5 days later.. In the right testicle, however, inoculated 25 days later with varicella virus no inclusions were found. The non-immune and immune sera used in the neutralization tests were obtained from the same patient; the former on the 2nd day of the disease, the latter on the 14th. The non-immune serum mixed with virus did not prevent the formation of nuclear inclusions in the testicles of Monkey S. On the other hand, the immune serum inhibited the production of inclusion bodies in the testicles of Monkey T.

Experiment 6.—Monkeys U and V; green. April 6, 1926. 5 lesions, vesicles and papules, were removed from a varicella patient, Case 30, within 48 hours after the appearance of the eruption, and emulsified. The volume of the emulsion was made up to 0.5 cc. with Locke's solution. Patient 30 also supplied the non-immune serum. The immune serum was obtained from Case 29 on the 22nd day after the onset of the disease. Both sera were fresh and not inactivated. 0.25 cc. of each serum was mixed with equal amounts of the virus emulsion. The mixtures were injected immediately into 2 green monkeys as follows: Monkey U, 0.25 cc. of virus and immune serum in each testicle; Monkey V, 0.25 cc. of virus and non-immune serum in each testicle. In addition to the above injections, the monkeys received intraperitoneally 3 cc. of the immune and non-immune sera respectively. The monkeys were castrated 5 days later and a search for the presence of nuclear inclusions in the testicles was made in the usual way.

Eosin-staining nuclear inclusions were found in both of Monkey V's testicles inoculated with a mixture of virus and non-immune serum. On the other hand, inclusion bodies were not seen in Monkey U's testicles inoculated with a mixture of virus and immune serum.

The results of the six experiments described above are summarized in Table I.

TABLE I.

Summary of the Results of Reinoculation and Neutralization Tests.

Experiment	Monkey	State of monkey	Inoculum	Nuclear inclusions.
1	L	Normal	Virus + convalescent serum	−
	M	Normal	Virus + non-immune serum	+
2	N	Normal	Virus + convalescent serum	−
	O	Normal	Virus + non-immune serum	−
3	P	Normal	Virus + convalescent serum	−
	Q	Normal	Virus + non-immune serum	+
4	A	Actively immunized	Virus	−
	R	Normal	Virus	+
5	R	Actively immunized	Virus	−
	T	Normal	Virus + convalescent serum	−
	S	Normal	Virus + non-immune serum	+
6	U	Normal	Virus + convalescent serum	−
	V	Normal	Virus + non-immune serum	+

\+ indicates presence of nuclear inclusions in testicles.
− " absence " " " " " "

DISCUSSION.

Neutralization and reinoculation tests are the accepted means employed to identify viruses and have been extensively used in investigations concerning vaccine virus (5), variola virus (5), poliomyelitis virus (6), Virus III (7–9), herpes virus (10, 11), and others. Technical details of the tests may vary somewhat with each virus, yet in every instance the usefulness of the tests is dependent either upon the specific virucidal properties of an immune serum or upon a specific refractory state of an immune animal.

Many viruses produce characteristic macroscopic lesions in animals, or cause marked changes in their condition. Such alterations and lesions serve as indications of virus activity. Under proper con-

ditions the occurrence of these changes is specifically prevented either by mixing the virus with a homologous immune serum prior to the inoculation of the animal or by injecting the virus alone into an animal previously immunized by means of the same virus. In addition to the characteristic macroscopic lesions already mentioned, many viruses also produce equally characteristic microscopic changes as indicated by the presence of inclusion bodies in the nuclei and cytoplasm of injured cells. Sometimes, however, the microscopic changes are the only manifestation of the presence of a virus (1, 12). When such a condition arises, there is no obvious reason why the microscopic changes, inclusion bodies, should not be used as guides or indicators with the same degree of readiness as that with which the macroscopic lesions are employed.

Many workers believe that the eosin-staining nuclear inclusions are the manifestations of the presence of certain filterable viruses, including the virus of varicella. Consequently at the time of the previous report (1) there were grounds for the belief that the nuclear inclusions found in the monkeys' testicles inoculated with emulsified varicella papules and vesicles were produced by the action of varicella virus. Proof of this, however, was obtained only recently by means of the experiments reported above, and consists, in brief, of the following considerations. Nuclear inclusions were not found in monkeys' testicles inoculated with a mixture of varicella virus and convalescent varicella serum. On the other hand, they were found in testicles inoculated with a mixture of virus and non-immune serum collected from varicella patients early in the disease. Furthermore, the inoculation of one testicle with varicella virus prevented the formation of nuclear inclusions in the other one when it was inoculated at a later date with the same virus.

So far as is known, the evidence presented in this paper is the only definite proof on record that experimental animals are susceptible in any way to the action of varicella virus.

CONCLUSION.

The eosin-staining nuclear inclusions found in the monkeys' testicles inoculated with emulsified tissue of human chicken-pox lesions are specifically associated with the virus of varicella.

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PHYSICAL, CHEMICAL, AND BIOLOGICAL STUDIES ON THE VIRUS OF VESICULAR STOMATITIS OF HORSES.

Comparison with the Virus of Foot-and-Mouth Disease.

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In an earlier paper[1] attention was drawn to the resemblances between the effects produced by the incitant of foot-and-mouth disease and of vesicular stomatitis. The characteristics to be especially mentioned are: propagation in guinea pigs in continuous series,[2] the correspondence of the experimental disease produced, and the possibility of transmitting vesicular stomatitis to cattle and swine. Such slight discrepancies as the infrequency of secondary lesions were ascribed to variability of strains of the virus. The two viruses were found to show parallel responses to selective filterability. On the other hand, certain clear-cut distinctions were noted, such as want of cross-immunity in guinea pigs, cattle, and swine, and the failure of the horse to respond to inoculation with the virus of foot-and-mouth disease, while being highly susceptible to the virus of vesicular stomatitis.

The present article deals with experiments on the physical, chemical, and biological characters of the virus of vesicular stomatitis, which may serve as a basis for wider comparison with that of foot-and-mouth disease, and at the same time provide data bearing on taxonomy. The latter subject has received, until the present, scant attention.

[1] Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1926, lxx (N.S. xxiii), 147. The Report of the Commission to Study Foot-and-Mouth Disease, to be published by the United States Bureau of Animal Industry should also be consulted.

[2] This has also been done with vesicular stomatitis virus by Cotton, W. E., *J. Am. Vet. Med. Assn.*, 1926, lxix (N.S. xxii), 313.

Description of the Strain of Virus Employed.

Source of Material.—Through the kindness of Dr. John R. Mohler, Chief of the United States Bureau of Animal Industry, and of Dr. W. E. Cotton, Assistant Superintendent of the Bureau's Experiment Station at Bethesda, Maryland, a sample of virus designated as that of vesicular stomatitis was received early in October, 1926. The specimen consisted of lingual vesicle coverings of a cow at the experiment station, which was inoculated in turn with material collected from a New Jersey cow ill with the disease. An extensive epidemic of vesicular stomatitis was raging at the time in northern New Jersey, and in the vicinity of Port Jervis, New York. The virus was active, for Dr. Cotton reproduced the disease in test horses in 20 hours. The material was sent in 50 per cent glycerol. A fragment 2 × 4 mm. was removed from the glycerol, washed in phosphate buffer at pH = 7.5,[3] (ground with sterile sand, and suspended in the buffer solution. About 0.1 cc. of this was injected by the method described[4] intradermally in the posterior pads of two guinea pigs. After 48 hours both animals showed the typical primary, or inoculation vesicles of experimental vesicular stomatitis, already described.[1] Up to the present the virus has been propagated in guinea pigs through at least 90 consecutive passages.

The clinical course of the affection in these animals was a counterpart of experimental foot-and-mouth disease. Secondary vesicles appeared in uninoculated pads in about half the animals; none was observed in the mouth or on the tongue. In these respects, we confirmed our earlier observations.[1] But the irregular occurrence of secondary lesions produced by this strain of virus does not indicate an essential difference between it and the virus of foot-and-mouth disease. We have already stated[1] that certain samples of foot-and-mouth disease virus may act in this manner in guinea pigs or in cattle.

In general, it may be stated that, apart from the likeness of the clinical course, other effects in guinea pigs of this strain of vesicular stomatitis virus are identical with those of the virus of foot-and-

[3] Phosphate buffer, as mentioned here and elsewhere in this article, is made by adding 2.5 gm. of potassium acid phosphate (KH_2PO_4) to a liter of distilled water. The solution is then adjusted to the desired hydrogen ion concentration by means of potassium or sodium hydroxide. It is of utmost importance to readjust the material immediately before use, for sterilization renders it acid.

[4] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673, and the Bureau Report.[1]

mouth disease.[5] For example, the mortality rate among 450 guinea pigs showing vesicular stomatitis was about 1 per cent; the affection is also practically non-lethal. The virus was free from constant, visible microorganisms, and on two occasions, when secondary, chance, microscopic bacteria were found admixed with the virus, they could be removed by Berkefeld filtration without injury to the specifically active agent. On the other hand, after inducing its specific effect, the latter invited invasion of ordinary bacteria—a character common to filter-passing viruses generally—so that a vesicle turned pustule on the 3rd to 4th day. Furthermore, in about 95 per cent of the animals the period of incubation was 18 to 48 hours, and the period became shorter and the severity of the disease increased as the concentration of the virus was augmented. Moreover, blood withdrawn 20 to 24 hours after intradermal inoculation was active. Aspirated vesicular contents showed the presence of virus in greatest concentration when this material was obtained from lesions up to 24 hours old. From this time to 72 hours a gradual diminution in virulence took place until after 3 days, when the vesicular contents, or ground infected pad tissues, were only exceptionally active. Finally, resistance of recovered animals to reinoculation with active material from the same source was marked, and the immunity lasted for at least $4\frac{1}{2}$ months.

Hence this sample of the virus of vesicular stomatitis is similar to the one already described[1] and induces in guinea pigs effects indistinguishable from those of the virus of foot-and-mouth disease.

Propagation in Rabbits.

The transfer of vesicular stomatitis to rabbits either has not been attempted, or has been unsuccessful and therefore not reported.

[5] The articles mentioned in Foot-note 12 should be consulted for a description of the effects of the virus of foot-and-mouth disease in guinea pigs. Since it is forbidden to work with the latter in the United States, no direct comparison could be made employing the same stock of normal guinea pigs, and the same laboratory conditions in the case of both viruses. For this reason no cross-immunity tests could be made here, although the results of a wide experience with another strain of the virus of vesicular stomatitis have already been reported.[1]

The rabbit appeared important for making additional tests on the above two viruses, and, incidentally, on another virus, namely, that of febrile herpes.

Comparison with Foot-and-Mouth Disease.—Gins and Fortner[6] found that rabbits could be infected with guinea pig virus by scarification of the mucous membrane of the inner lip surface. Vesicles appeared, and the contained fluid was in turn active for normal rabbits or for guinea pigs. Sixteen consecutive rabbit passages were thus effected. Nicolau and Galloway,[7] employing guinea pig virus, were able to induce local vesicles after intralingual injections. In neither case was there evidence of secondary lesions nor of generalization of the vesicular process.

We studied the susceptibility of rabbits to vesicular stomatitis by injecting the virus in the brain, cornea, and buccal mucous membranes.

The rabbits failed to show any untoward effect after intracranial injection of the active Berkefeld V filtrate obtained from guinea pig pads, which was in the ninth guinea pig passage. In another test, virus was employed which was adapted to rabbits by successful corneal inoculation. The materia of the third, fourth, and fifth corneal passages was kept in 50 per cent glycerol. The glycerol was renewed weekly three times, the corneal tissue was then removed, washed, suspended in saline solution, and injected intracranially. While the injected material was active for the pads of guinea pigs, it failed to affect rabbits upon intracranial inoculation.

It appears, therefore, that the virus of vesicular stomatitis, like that of foot-and-mouth disease,[8] is non-neurotropic. Furthermore, in sharp contrast to the effects of herpetic virus, none of the guinea pigs injected with the virus of vesicular stomatitis showed microscopic evidences of damage to the nervous tissues.

On the other hand, the rabbit reacts specifically to corneal inoculations.[9] Beginning with filtered suspensions of infected pads of the

[6] Gins, H. A., and Fortner, J., *Berl. tierärztl. Woch.*, 1926, xlii, 89; *Centr. Bakt., 1. Abt., Ref.*, 1925, lxxviii, 576.

[7] Nicolau, S., and Galloway, I.-A., *Compt. rend. Soc. biol.*, 1925, xciii, 1283.

[8] For the non-neurotropic effects of foot-and-mouth disease virus consult Levaditi, C., Alberta-Lorente, R., and Galloway, I., *Compt. rend. Soc. biol.*, 1926, xcv, 387.

[9] For mode of inoculation see Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 233.

guinea pig, and later employing the ground corneal scrapings, all of thirteen rabbits, during five consecutive passages, showed typical vesicles along the lines of incision of the cocainized cornea. The lesions appeared from 2 to 4 days after application of the virus and were accompanied by conjunctival inflammation. The vesicles coalesced as a rule, and in two rabbits left small, localized opacities. There was no fever at any time. After 7 days healing began, so that within 2 weeks the eye returned to normal. 10 days later the recovered corneæ were refractory to reinoculation. As little or no work was done in this way with foot-and-mouth disease virus, no comparisons can be made.

As is the case with foot-and-mouth disease virus, scarification of the inner surface of the lips and cheek of the rabbit, and application of filtered suspensions of infected pads of guinea pigs led, within 24 to 48 hours, to distinct, localized vesicles. The histopathology of the lesion is similar to that of the guinea pig pads from experimental vesicular stomatitis and foot-and-mouth disease.

The rabbit, therefore, is susceptible to the virus of vesicular stomatitis, and where comparisons can be made with that of foot-and-mouth disease in the same animal, its effects appear to be identical. The rabbit and the guinea pig are epitheliotropic, but not neurotropic toward the viruses.

Comparison with Herpes.—A limited number of experiments was made with the H. F. strain of herpetic virus described by Flexner and Amoss.[9] This virus, injected intracranially in a rabbit, produced the typical cerebral symptoms and death in 4 days. A portion of the base of the brain was suspended in saline solution equal to a 10 per cent suspension, and of this 0.1 cc. was injected intradermally in the posterior pads of three guinea pigs. In general, the effects were similar to those described by Gildemeister and Herzberg.[10] Edema of the pads was noted, and this persisted for about 4 days, when vesicles appeared. These were often pustular from the start, coalesced, tended to necroses, and lasted for about a week. In addition the frequent occurrence of gangrene in the phalangeal and metatarsal areas, and in all of ten guinea pigs injected through three consecutive passages, retention of urine and feces, and paralyses of the posterior extremities were present. The nervous symptoms first appeared about 5 to 6 days after injection. Only one of the ten animals survived. No secondary vesicles were observed.

[10] Gildemeister, E., and Herzberg, K., *Deutsch. med. Woch.*, 1925, li, 97.

These comparisons show the herpetic virus to be distinct from the viruses of vesicular stomatitis and of foot-and-mouth disease. These differences are emphasized by the fact that guinea pigs recovered from experimental vesicular stomatitis are susceptible to the herpetic virus and *vice versa*.

The reactions of rabbits to corneal inoculation of the herpetic and of the vesicular stomatitis virus differ. In the former the affection induced is much more severe locally, and encephalitic signs may become evident; in the latter, the lesions in the eye are ordinarily mild, tend to complete and rapid recovery, and no nervous manifestations are observed. Cross-immunity is absent in the recovered animals.

Histopathology of Experimental Vesicular Stomatitis of the Guinea Pig.

In pads of guinea pigs inoculated intradermally with filtered or unfiltered material containing the virus of vesicular stomatitis, the first changes noted are swelling and thickening of the epidermis and derma, as a result of edema and cellular infiltration. The infiltrating cells consist mainly of polymorphonuclear neutrophils (the so called pseudo-eosinophils) and less numerous, monocytes (or macrophages or endothelial leucocytes). After 18 to 24 hours vesicles appear between the epithelial layer and corium, and between the horny and Malpighian strata. These layers are filled with serum at first, but soon cells, especially the neutrophils, invade the fluid. In the corium, however, the vesicles may be filled with blood, with an eventual greater cellular infiltration. At this stage occur active mitosis and striking intranuclear changes, to be described immediately. After 48 to 72 hours, necrosis of epithelial cells is observed and retrogression begins; the vesicles are filled with multinucleated cells and later with granular material and droplets of various sizes. They now begin to contract and to dry. After 3 to 4 days, the horny layer exfoliates, a large number of neutrophils is present, and with this a proliferation of epithelial cells about the site of the vesicle. Thereafter healing begins and the lesion is covered with a scab composed of leucocytes and epithelial cells. After the 8th day, as a rule, the cells of the epidermis appear normal for the most part, and the vesicles are replaced by a highly vascularized granulation tissue.

The nuclear changes of the epithelial cells in the Malpighian layer,

in infiltrating cells of the corium and of the vesicles consist in a condensation of the chromatin about the wall, thus leaving only a shadow of nuclear structure which is surrounded by a darker staining, denser membrane. The nucleoli and nucleolar degenerated particles are stained bluish by Giemsa's or the eosin-methylene blue methods. Within the membrane may be seen, in specimens stained by Giemsa's method, one or more perfectly rounded, light pinkish staining bodies, about 1.5 microns in diameter. Some are smaller, but rarely are there any larger. These changes are most marked in lesions 24 hours old, and are comparable to the so called inclusion bodies described by Gins[11] as characteristic of foot-and-mouth disease. In lesions which are 48 hours old, a large number of somewhat different bodies are noted. They are round or oval and vary in diameter from 2 microns to a size large enough to fill almost the entire nucleus with the exception of a narrow, clear zone between the body and nuclear membrane. With Giemsa's or eosin-methylene blue stains, they appear pink to red in contradistinction to the blue nuclear membrane, and, as a rule, lie on a clear background. Some nuclei may contain from two to four of the smaller sized bodies. In general, they are similar to but not necessarily identical with the intranuclear inclusions which characterize certain other filter-passing viruses.

To summarize, the histopathology of experimental vesicular stomatitis is identical with that of experimental foot-and-mouth disease, described by several observers, notably by Gins,[11] and by Levaditi and his coworkers.[8] Furthermore, we have found indistinguishable pathological conditions in foot-and-mouth disease, not only of guinea pigs, but also of cattle and swine. Finally, it is important to note that the virus of vesicular stomatitis can be classified as one of a group of ultramicroscopic agents, the effects of which are characterized by the presence of peculiar intranuclear changes. Further studies by Dr. Rivers on their significance are in progress.

Titration of Virus.[12]

The virus of vesicular stomatitis, as it exists in ground infected guinea pig pads, or in aspirated vesicular contents, could be diluted 1:10,000.000, but not higher,

[11] Gins, H. A., *Centr. Bakt., 1. Abt., Orig.*, 1922, lxxxviii, 265.

[12] In this, as in other experiments, the technical procedures of experiments are omitted. For details of methods, see Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673, 685, 815, 833, and the Bureau Report.[1]

and still show activity. In filtered material a 1:10,000,000 dilution also induced the experimental disease in guinea pigs. In the blood of this animal, withdrawn 20 to 24 hours after pad inoculation, the virus was present in much lower concentration: the limit of infectiousness being 1:2000 to 1: 200,000.

According to these tests which show the limit of infectiousness at 1:10,000,000 in filtered or unfiltered material, the virus of vesicular stomatitis corresponds in activity to that of foot-and-mouth disease.[4]

Failure of Sedimentation of the Virus.

Centrifugation, at 3700 revolutions a minute for 2 hours, of material containing the virus of vesicular stomatitis failed to bring about its sedimentation. In graded dilutions of 1:30 to 1:300,000, the topmost and the lowest layers behaved in all instances alike. In this respect also the two viruses are similar.[4]

Selective Filtration through Chamberland Bougies.

In an earlier paper[1] it was stated that the behavior of the virus of vesicular stomatitis on filtration paralleled that of the virus of foot-and-mouth disease. Both passed readily through Seitz' asbestos discs and Berkefeld V candles, but not always through Berkefeld N filters. The same was true in the case of Chamberland bougies, both viruses passing through the L 3 and L 7, but usually not through the L 11 type.[13] Hence it was concluded that the two viruses had the same tendency to adsorption in the walls of denser, electronegative filters.

Because of the importance of the L 11 bougie in differential filtration, the tests have been repeated with the particular sample of vesicular stomatitis virus at hand. Seven trials were made with the active material suspended in phosphate buffer at pH = 7.5 and at 8.5. At pH = 7.5, the virus passed through only one of three bougies but a portion of the same active material at pH = 8.5 traversed all of the four L 11 filters employed.

In the case of the virus of foot-and-mouth disease, a similar phenomenon was interpreted as evidence that the incitant is electropositive—a conclusion which was confirmed by the behavior of the virus on cataphoresis and by the determination of its isoelectric range at pH = about 8. Although cataphoresis tests have not been

[13] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 685.

made with the virus of vesicular stomatitis, the indications are that it conforms with that of foot-and-mouth disease in respect to magnitude, charge, and isoelectric range.

Effect of Hydrogen Ion Concentration on Viability.

It has already been shown by Bedson and Maitland,[14] by Stockman and Minett,[15] and by us[16] that the virus of foot-and-mouth disease survives longest in a medium of which the hydrogen ion concentration is at pH = 7.5 to 7.6, and that viability is diminished considerably by slight variations above or below this narrow range. The same conditions apply to the virus of vesicular stomatitis. For example, the latter remained alive in phosphate buffer at pH = 7.2 and 7.5 to 7.6 for at least 52 hours at 37°C., but was inactive at pH = 6.8 and 8.0 at this time. After 100 hours, however, only the material at pH = 7.5 to 7.6 was active. The precise end-point was not determined.

Resistance to Chemicals.

The virus of foot-and-mouth disease is highly resistant to chemicals, such as alcohol, ether, chloroform, glycerol, and to many so called antiseptics, such as bichloride of mercury, cresol, phenol, etc.[14,15,17] We have pointed out that this remarkable resistance is due to the fact that the chemicals coagulate the proteins of the medium in which the virus is suspended and these in turn prevent direct contact of the virus with the reagents. If the periodic phenomenon attending such processes is considered and coagulation is prevented, the virus is made to come directly under the influence of the chemicals. Under these conditions, it is more sensitive to destruction by them than is staphylococcus. Moreover, the virus of foot-and-mouth disease is destroyed as rapidly as staphylococcus, or even more rapidly, by substances

[14] Bedson, S. P., and Maitland, H. B., *J. Comp. Path. and Therap.*, 1925, xxxviii, 229.

[15] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

[16] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 833, and the Bureau Report.[1]

[17] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 815, and the Bureau Report.[1]

such as sodium hydroxide (1 to 2 per cent) or antiformin (1 per cent), which do not form coagula.

A similar series of tests was made with the virus of vesicular stomatitis in which we selected as an example of the narcotic solvents, 60 per cent alcohol, and of other coagulating substances, bichloride of mercury (1:1000), cresol (3 per cent), and phenol (1 per cent). Of the non-coagulating chemicals sodium hydroxide (2 per cent) was chosen.[12]

The virus was still active after 24 hours in 60 per cent alcohol. But if sodium hydroxide (1:5000), in which it can survive for at least a day, was added, and coagulation thus prevented,[17] it was killed within 1 minute. In bichloride of mercury, cresol, and phenol, in the dilutions mentioned, active materials remained viable for at least 6 hours. Tests for longer periods were not made. In sodium hydroxide (2 per cent) the virus was killed within 1 minute. An additional test revealed that 3 per cent cresol containing 1 per cent sodium hydroxide also inactivated it within 1 minute.

The virus remained active for at least 4½ months in 50 per cent glycerol buffered at pH = 7.5, and kept at 4–6°C.

In so far as the resistance to chemicals is concerned, therefore, the virus of vesicular stomatitis resembles that of foot-and-mouth disease.[17]

Survival of the Virus Outside the Body.

It has been found[18] that in moist or palpably dried garden soil, the virus of foot-and-mouth disease survives for at least 25 days, and in hay for at least 1 month. Active materials derived from vesicular stomatitis also maintain their activity for a considerable time after leaving the body. Infected guinea pig vesicle coverings remained infectious for at least 31 days in garden soil kept at 4–6°C., or at 20°C., in a moist or in a palpably dried state.

Respiration of the Virus.

A study of the respiration of the virus of vesicular stomatitis was undertaken with the idea of obtaining information concerning its living character. The active agent could not, however, be separated in a pure state from respiring living tissues, nor could it maintain its

[18] Report of the Commission to Study Foot-and-Mouth Disease, to be published by the United States Bureau of Animal Industry.

life under the conditions imposed by the experiment. Such was also the case with the virus of foot-and-mouth disease.[18]

Effect of Ultra-Violet Light on Viability.

In contrast to the results of the tests on respiration are those of the following experiments, done with the collaboration of Dr. F. L. Gates, of The Rockefeller Institute, on the effects of different wave-lengths and energies of monochromatic ultra-violet light. Since no similar tests were made with the virus of foot-and-mouth disease, no comparison of the two viruses can be offered in this respect. The experiments are reported, however, as adding suggestive evidence on the relation of the incitant of vesicular stomatitis to living bacteria. This evidence was obtained by comparing the behavior of the virus with that of *Staphylococcus aureus* under similar exposure to ultra-violet irradiation.[19]

Aspirated vesicular contents diluted 1:10 in buffered broth at pH = 7.4 were employed as virus and a thin suspension of *Staphylococcus aureus* from an 18 hour broth culture as control. The surface of thin layers of 2 per cent agar buffered at pH = 7.4 in small Petri dishes was washed with each of these materials respectively. After exposure, the virus-agar was cut within the limits of the area of light penetration, ground in a mortar, suspended in phosphate buffer at pH = 7.5, and of this about 0.4 cc. was injected intradermally into the posterior pads of guinea pigs. An additional control of unexposed virus-agar strips of similar size was also used in each test. After exposure, the agar, seeded with staphylococci, was incubated overnight at 37°C., and the number of colonies appearing in the exposed areas was compared with that in like areas from the unexposed portion of the plates.

At lambda = 2675 Ångström units, with a total energy of from 512 to 540 ergs per sq. mm., all the staphylococci were killed; at the same wave-length and from 256 to 270 ergs per sq. mm., 87 to 97 per cent of the organisms were killed. In respect to the virus subjected to the greater energy all of five guinea pigs were negative after inoculation, and in the second instance in which the lesser energy was used only one of four guinea pigs showed the experimental disease. All of five guinea pigs injected with unexposed virus-agar (controls) revealed typical lesions.

At lambda = 3022 Ångström units, with a total energy of 23,300 to 29,900 ergs per sq. mm., 97 to 100 per cent of the staphylooocci were killed. The exposed

[19] Olitsky, P. K., and Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 431.

virus-agar inoculated into four guinea pigs failed to infect, but the unexposed virus-agar (control) induced the experimental disease in all of four animals.

At lambda = 3126 Ångström units with a total energy of 60,400 ergs per sq. mm., there was no visible effect on the staphylococci. The exposed virus-agar proved active in three of four guinea pigs, and the unexposed similar material, as a control, induced the experimental disease in all of four animals.

It is thus evident that the transmissibility of the virus of vesicular stomatitis is lost on exposure to the same wave-lengths and energies of monochromatic ultra-violet light that are bactericidal. Furthermore, at the limits of destructive action of ultra-violet light, the reaction of the virus parallels that of the microorganism. Since adsorption of specific energies is one index of chemical character, these parallel reactions suggest that the substance of the virus is similar in character and chemical constitution to bacterial protoplasm.

DISCUSSION.

In an earlier paper[1] reference was made to the similarity of the virus of vesicular stomatitis and of foot-and-mouth disease. Slight differences between them were ascribed to the variability of different strains. In the foregoing pages, additional evidence is presented to show their resemblance in several other reactions, physical, chemical, and biological. Furthermore, the clinical appearance of the diseases produced by the two viruses may be the same. Under field conditions and among cattle, the method employed heretofore in the United States for differential diagnosis has been to inject suspected material into a horse. If it reacted with typical vesicular lesions, a diagnosis of vesicular stomatitis was made; if it did not, the material was designated as having been derived from foot-and-mouth disease.[20]

There are, therefore, under consideration two viruses differing practically only in an absence of cross-immunity in recovered animals and in the resistance of the horse to one of them. In view of the non-cultivability of either virus, it is difficult to prove the precise relationship of one to the other. Yet, if a comparison be made with what exists among the types of foot-and-mouth disease virus itself or among known, cultivable bacteria, a close relationship between the two may be inferred. For example, there are at least two types of

[20] Mohler, J. R., *U. S. Dept. Agric., Dept. Circular 400*, 1926.

the active agent of foot-and-mouth disease, either of which shows no cross-immunity to the other in recovered animals. While all strains are generally active in cloven foot animals, there are some to which the guinea pig is resistant. Here, then, is a genus containing types which do not exhibit cross-immunity and show different pathogenic effects in a different species of animal. Among examples of similar behavior of cultivable microorganisms may be mentioned those dealt with in the recent studies of Tillett.[21] The rabbit which is susceptible to infection with Type I and Type II pneumococcus, is practically resistant to Type III. It is well known, moreover, that these different types do not show cross-immunity.

It appears, therefore, from the resemblance of the viruses of vesicular stomatitis and of foot-and-mouth disease, as demonstrated in this and in an earlier paper,[1] that their relationship is close. But this is an inference based on indirect evidence and is tentative until artificial cultivation of the viruses is obtained.

CONCLUSIONS.

A taxonomic study of the virus of vesicular stomatitis is presented along with evidence additional to that already reported[1] to show the similarity of this virus to that of foot-and-mouth disease. The connection of the two is discussed and the deduction drawn that their generic relationship is close. On the contrary, the differences between these two viruses and the herpetic are sufficiently marked to indicate a lack of generic connection among the three.

The results of a comparative study on the effects of particular wave-lengths and energies of monochromatic ultra-violet light on the virus and on *Staphylococcus aureus* reveal that the adsorption of specific energies by the two is parallel. Since the adsorption of specific energies is an index of chemical character, these experiments suggest that the virus is similar in character and chemical constitution to bacterial protoplasm.

[21] Tillett, W. S., *J. Exp. Med.*, 1927, xlv, 1093.

THE INFLUENCE OF OVARIECTOMY ON THE SPONTANEOUS OCCURRENCE OF MAMMARY CARCINOMAS IN MICE.*

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Lathrop and Loeb (1), working on strains of mice with a high percentage of spontaneously developing tumors, have shown that the castration of female mice below the age of 6 months leads to a marked decrease in the tumor incidence. In a later report Loeb (2) was able to confirm and enlarge his previous results.

According to Loeb an ovarian hormone regulates those tissue changes which lead to the development of breast tumors in mice. The relationship between this hormone and the development of tumors is quantitatively graded. If the quantity of hormone acting on the breast tissue exceeds a certain limit, cancer appears as frequently in castrated as in non-castrated animals. This is the case when the mice are operated at 8 to 10 months of age. If an intermediate amount of hormone has been allowed to act on the mammary gland, that is, if castration has been performed at an age of 5 to 7 months, the cancer rate is noticeably diminished and the tumors appear at a later age period than in non-operated controls. If, finally, the quantity of the hormone has been restricted still further by operating on the animals at a still younger age, tumors either do not appear at all or only in exceptional cases. Loeb concludes that the hereditary factors which determine the development of breast carcinomas in mice need the cooperation of a definite quantity of ovarian hormone, if cancer is to develop.

As far as the writer is aware, the experiments of Loeb have not been repeated. This may be due to the fact that strains of mice with a

* Presented before the nineteenth annual meeting of the American Association for Cancer Research.

high tumor incidence are rather difficult to obtain. Several years ago, Mr. Marsh, the biologist of this Institute, succeeded in evolving such a strain by selective crossing. Reports concerning the tumor incidence of this strain have been made on several occasions (3, 4). In eight inbred generations, covering a period of 5 years, the cancer rate and cancer age varied but little. Close to 94 per cent of all the breeding females that reached the cancer age developed breast carcinomas. Tumors appeared as early as 5 months and reached a maximum incidence at the age of 9 months.

It is necessary to use non-breeding females as controls for the castrated animals, since prevention of breeding decreases the percentage of tumors developing and increases the age at which tumors occur. Table I[1] indicates that of 42 non-breeding females living to tumor age, 33 developed spontaneous breast carcinoma. This corresponds to a tumor incidence of 78.5 per cent. In a previous experiment, 14 out of 18 non-breeding females developed tumors, which corresponds to an incidence of 77.7 per cent. The first tumors appeared later than in breeding females, namely at 10 to 11 months of age, while the highest tumor incidence occurred between 14 and 15 months of age.

Two series of experiments were made, but for reasons to be stated later only the second series is reported here in detail. In the first series groups of 20 to 30 female mice were castrated at 2, 3, 4, 5, and 6 months of age. The operation was performed by the abdominal route, consisting of the removal of the entire capsule containing the ovary, the Fallopian tube, and the tip of the uterus horn. Males were kept in the same cages as the operated females, but a pregnancy was never observed. An autopsy was performed on most animals that died spontaneously and on all animals that developed a tumor.

Surprisingly enough, it was found that about 15 per cent of the castrated animals showed one and in a few cases even two ovaries at the site of the previous operation. Upon microscopic examination some of these ovaries were in a state of degeneration or even calcified, others, however, showed follicles and corpora lutea. The possibility, that the ovaries had been incompletely removed, seemed very remote. It was, therefore, concluded that a regeneration of ovarial tissue had taken place. This was amply confirmed by a paper by Davenport (5) that appeared later.

[1] Compiled from the statistics kindly furnished to us by Mr. Marsh.

This author made a detailed study of the conditions under which ovaries regenerate in mice. Regeneration is less apt to occur when the capsule, tube, and part of the fat body surrounding the ovary are removed than when the ovary alone is removed by cutting its stalk and picking it out of the capsule. With the former method of re-

TABLE I.

Monthly Expectancy of Tumor Incidence in Non-Breeding Female Mice (Strain 3).

Based on 42 mice living to tumor age, of which 33 developed tumors. Incidence: 78.5 per cent. Females isolated at 1 month of age, one in each cage. Remained isolated throughout life. The food consisted of dog bread.

Age	No. of tumor mice	Per cent of 42 tumored during each month	Total per cent tumor mice at end of each month
mos.			
1–10	0	0	0
11	2	4.76	4.76
12	2	4.76	9.52
12½	2	4.76	14.28
13	1	2.38	16.66
13½	1	2.38	19.04
14	4	9.52	28.56
14½	1	2.38	30.94
15	6	14.28	45.22
15½	1	2.38	47.60
16	2	4.76	52.36
18	2	4.76	57.12
19	2	4.76	61.88
20	5	11.90	73.78
20½	1	2.38	76.16
21	1	2.38	78.54
	33	78.54	

The 9 mice without mammary tumors lived 7 to 18 months, average 12.5 months. One of these mice developed a spindle cell sarcoma. Reckoned as a clean mouse above.

moval, regeneration took place in 18 per cent of the cases, but when the ovary alone was removed, regeneration was observed in 64 per cent of the operated animals. Regeneration may take place as early as 1 and as late as 6 months after the operation. The age had no demonstrable effect upon the chance of regeneration, except when the mice

were very young. In several cases two ovaries regenerated in the place of one or the new ovary arose at a point ½ cm. distant from the position of the former ovary.

There seems no doubt that the ovary of the mouse may regenerate from the peritoneum under favorable conditions. Prior to Davenport, Castle and Phillips (6) observed ovarian regeneration in 11 and pregnancy in 3 out of 141 operated guinea pigs.

Interesting as it may seem that ovaries regenerate in mice, it was a rather unfortunate occurrence from the point of view from which these experiments were undertaken. Animals that showed ovaries upon postmortem examination, whether they were normal or tumor mice, had to be ruled out. When these were omitted, there remained 49 animals that were still alive at an age of 19 months. 44 of these mice remained free from tumors until they died of old age or of intercurrent diseases. 5 animals out of these 49 developed tumors, 1 at 18½ months, 1 at 19 months, 2 at 20 months, and 1 at 21 months. This gives a tumor incidence of 10 per cent, as compared with 78.5 per cent for the non-breeding controls. There seems no doubt that ovariectomy performed between 2 to 6 months of age reduces the tumor incidence and increases the tumor age.

In the second series of experiments the control of the experimental conditions was very satisfactory. The underlying idea was to see if the occurrence of tumors could be prevented altogether by operating on the animals at a very young age. 100 mice were castrated between 15 and 22 days of age, that is, immediately after weaning. The ovaries were exposed by the lumbar route and the entire fat body surrounding the ovary together with a portion of the uterus horn were removed. Beginning with 6 months, the mice were examined for tumors regularly once a week throughout their life time. Great care was taken to autopsy as many mice as possible.

In no case did the postmortem examination reveal the presence of ovarian tissue. However, from the experience gained in the first series of experiments it seemed unwise to rely on the autopsy alone. The absence of ovaries at the time of death was not considered proof enough that no regeneration had taken place, since most of the animals were very old when they were examined. A regenerated ovary might have disappeared again through absorption. Use was made of the vaginal smear method of Stockard and Papanicolaou (7), which indi-

cates in a definite way whether or not ovarian activity is present. In the mouse the estrus cycle lasts from 4 to 6 days. At the height of the estrus the vaginal smear is characterized by the presence of large cornified cells without a nucleus and by the complete absence of leucocytes. In the resting stage of the estrus cycle a large number of leucocytes are present and some small, nucleated epithelial cells, while the characteristic cornified cells are missing. For 1 consecutive

TABLE II.

Absence of Spontaneous Mammary Carcinomas in Micé Ovariectomized between 15 and 22 Days of Age.

Most of the operated mice belonged to Strain 3, which has a tumor incidence of 78.5 per cent. A few mice were from Hybrid Strain 1, which has a tumor incidence of 50 per cent.

Age	No. of mice alive	Remarks
mos.		
6	94	
10	86	
12	84	
14	79	
16	73	1 killed at 15 mos. Spindle cell sarcoma of fore leg.
		1 killed at 15½ mos. Spindle cell sarcoma of tail.
18	72	
20	60	
22	37	1 killed at 21 mos. Spindle cell sarcoma of abdominal wall.
24	14	
26	5	
28	1	

week vaginal smears were made on each mouse 2, 4, and 6 months after the operation. Only one mouse with a positive vaginal smear was picked out in this way. This animal through an oversight proved not to have been operated upon. The fact that the vaginal smears were all negative makes it certain that the mice of this series were entirely free from ovarian activity throughout their life time.

The result was that none of these mice developed a spontaneous

adenocarcinoma of the breast. Table II contains a detailed record of this series of experiments. From 86 mice that were still alive at 10 months of age, that is, at the time that the first tumors appeared in the non-breeding controls, 60 mice reached an age of 20 months. At 20 months of age 73.8 per cent of the control mice had developed tumors, while none of the operated animals had shown a breast tumor so far. At 21 months the last control mouse had died, while a larger number of operated mice continued to live up to 24 months and more. The chance to develop a tumor was, therefore, greater in the operated mice than in the control mice. Nevertheless, all the operated animals remained free from breast carcinomas. This remarkable effect of the ovarian hormone on the development of spontaneous breast tumors in mice seems to be a specific one, since the absence of the ovaries did not prevent the occurrence of other types of tumors. It will be noted in Table II that 3 of the castrated mice developed spindle cell sarcomas. A low incidence of spontaneous sarcomas has been observed previously in this strain of mice and is, therefore, nothing abnormal. The conclusion seems justified that the lack of breast tumors in spayed mice is not due to some indirect effect of castration. The body changes produced by castration are not such that they lead to a resistance against spontaneous tumors in general. Only the tumors arising from the mammary gland, a tissue that is directly influenced by the sex hormone, are prevented from developing. Another point of interest is that the prevention of breeding prolongs the life time of the mouse and that mice spayed at an early age live still longer than the non-breeding controls.

DISCUSSION.

Loeb interpreted his results on the basis of the interrelation between the ovary and the mammary gland. Since that time our knowledge of the ovarian function has been increased considerably, chiefly through the isolation of a potent ovarian hormone from the liquor folliculi by Allen and Doisy (8). If the ovaries are removed before sexual maturity is reached, the mammary tissue remains in an undeveloped state throughout the life of the animal, similar to the undifferentiated state of the breast tissue of the male animal. Our experiments indicate that a malignant change does not occur in this

undifferentiated tissue. Nevertheless, these animals must be regarded as potential tumor bearers, since they have inherited an organ disposition to develop tumors from previous generations. But the hereditary organ disposition alone does not lead to carcinoma of the mammary gland. The cooperation of the ovarian hormone is necessary, first, to cause the full development of the breast and second, to induce a periodic stimulation of the mammary tissue. During the life of the adult female the estrus cycle, which lasts from 4 to 6 days, provides a periodic stimulation of the breast tissue. One can calculate that a mouse has about 70 to 80 estrus cycles during life. If castration has been performed at 2 to 5 months of age, that is, after 3 to 20 estrus cycles have taken place, the occurrence of tumors is greatly reduced but not entirely suppressed. Castration after 6 to 7 months, that is, after 30 periodic enlargements of the mammary gland, has no decided influence on the tumor incidence. The tumors appear at the same rate as in the animals that had intact ovaries throughout their life. The point of interest is that a relatively short period of functional activity of the mammary gland leads to those tissue changes that result later on in the development of carcinoma. Equally striking is the long latent period between the ovarian stimulus that leads to cancer and the onset of the cancerous change itself.

When the tumor is once established, the ovarian hormone seems to be without influence on the rate of growth of the tumor. This is shown by the fact that spontaneous tumors in previously castrated animals do not grow less rapidly than tumors developing in normal animals. Even a surplus of ovarian hormone does not increase the rate of growth of mammary carcinoma, as may be judged from experiments that have been made with the ovarian hormone of Allen and Doisy. This hormone, when injected into spayed animals, produces the typical estrus changes in the genital organs and causes an enlargement of the milk ducts of the breast with proliferation of the glandular elements. It seemed of interest to investigate, whether this hormone would also have a growth-promoting effect on the adenocarcinoma which arises from the normal glandular elements of the breast. 50 normal females with either spontaneous or transplanted breast carcinoma received 4 to 10 hormone injections at 5 day intervals. The potency of the hormone had previously been tested on spayed animals.

However, no definite influence of this surplus of ovarian hormone on the rapidity of growth of the tumors could be observed.

It is a well known fact that the males belonging to inbred strains of mice with a high tumor incidence, do not show mammary carcinoma. Even though the males transmit the hereditary factors, they remain free from tumors, because the mammary tissue remains undeveloped in analogy to the female mice castrated before puberty, which also remained free from tumors. Loeb tried to produce breast tumors in males by transplanting ovaries into them, but obtained negative results. The author also obtained negative results by this procedure. Males belonging to the high tumor strain were castrated at an early age. When they were 2 and 6 months old, two ovaries of adult females were transplanted into the peritoneal cavity of each mouse. Most of the animals reached an old age, but breast tumors were not observed. It was also tried to produce breast tumors in males by the injection of the ovarian hormone. The hormone was prepared from the liquor folliculi of hogs according to the original prescription of Allen and Doisy (8). The chief difficulty was that at that time the only suitable solvent for the hormone was oil. The injections could not be extended over 10 weeks without causing infiltrations and general impairment of the health of the animals. Many months after the injections had been stopped, unabsorbed oil was still present in the subcutaneous tissue. A positive result could only be expected, if the injections could be continued throughout life. One of the animals developed a sarcoma adjacent to an oil cyst, the other animals remained free from tumors. Recently it has been possible to prepare the hormone in a water-soluble form. The experiments on male animals are being repeated, since it would be of considerable importance to produce breast tumors in male animals experimentally.

SUMMARY AND CONCLUSIONS.

1. Castration of female mice between 15 and 22 days of age entirely prevented the occurrence of spontaneous adenocarcinoma of the breast, while the non-breeding control mice of the same strain showed a tumor incidence of 78.5 per cent. 3 of the spayed animals developed a spontaneous spindle cell sarcoma. This indicates that the influence

of the ovary on the development of breast carcinomas is a specific one, since ovariectomy does not lead to a resistance against other types of spontaneous tumors.

2. Castration between 2 and 6 months of age led to a marked reduction of the tumor incidence, but did not entirely prevent the occurrence of mammary tumors.

3. It is concluded that the spontaneous mammary carcinoma of the mouse is due to a hereditary organ disposition, which remains latent in the absence of ovarian function, but which becomes manifest when a certain amount of ovarian hormone, corresponding to 5 to 30 estrus cycles, has acted on the breast tissue.

The author wishes to thank Dr. B. T. Simpson, Director of this Institute, for his kind interest in this work, Mr. M. C. Marsh for supplying the mice, and Miss Cora Geisler for valuable technical assistance.

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FURTHER OBSERVATIONS CONCERNING GROWTH REQUIREMENTS OF HEMOPHILIC BACILLI.

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Hemophilic bacilli form a heterogeneous group of bacteria. Observations concerning their growth requirements and the hemolysis caused by some strains have been reported. By means of these observations a subdivision of this group of organisms has become possible.

Pfeiffer (1), 1893, first described *B. influenzæ*. He was unable to cultivate the bacillus in the absence of blood pigment. Grassberger (2), 1898, reported that Pfeiffer's bacillus in symbiosis with *Staphylococcus aureus* was able to multiply on media containing hematin. Olsen (3), 1920, showed that growth of *B. influenzæ* occurred only on media giving a positive peroxidase reaction. Fildes (4), 1920, obtained satisfactory growth of Pfeiffer's bacillus on media enriched by means of a peptic digest of blood. Later, in 1921, he also showed (5) that in this digest each of two factors was essential for the growth of the organism; one was present in the clear supernatant fluid, the other occurred in the brown hematin deposit. Avery and Thjötta (6, 7), 1921, in a series of papers, reported that two accessory factors are necessary for the cultivation of influenza bacilli. These factors were designated as X and V. The V substance is relatively heat-labile and is found in blood, in cultures of many bacteria, and in yeast and vegetable cells. The X substance is heat-stable, gives a positive peroxidase test, and is found in blood and raw potatoes. Rivers and Poole (8), 1921, showed that two accessory factors are essential for the growth of influenza bacilli; one is present in filter-sterilized yeast extract, the other in autoclaved extract of blood. Rivers (9, 10), 1922, reported that Friedberger's *B. hemoglobinophilus canis* requires only the X accessory factor, and that from patients 2 strains of bacilli had been obtained that require only the V accessory factor. The strains requiring only V factor were designated *B. parainfluenzæ*. Rivers and Bayne-Jones (11), 1923, obtained from throats of cats 6 strains of influenza-like bacilli that required only the V factor.

Pritchett and Stillman (12), 1919, described a Gram-negative, aerobic, nonmotile, hemophilic bacillus and designated it as Bacillus "X." Stillman and Bourn (13), 1920, reported further observations concerning the characteristics of Bacillus "X." Rivers (14), 1921, suggested that these organisms be called hemolytic influenza bacilli. Rivers (10), 1922, on testing a hemolytic strain found that

it required both X and V growth accessory factors. Fildes (15), 1924, reported that 13 of 14 strains of hemolytic influenza bacilli required only the V factor. The remaining strain, however, required both X and V. The former strains grew on ordinary media in symbiosis with staphylococci, and also with *B. hemoglobinophilus canis* on media containing the X factor. This fact indicated to Fildes that *B. hemoglobinophilus canis* synthesizes the V factor.

Particular interest in the hemophilic bacilli was again aroused by the occurrence in New York during the spring of 1926 of a respiratory infection resembling mild influenza. Attempts were made to determine (1) how frequently hemophilic bacilli occur in the upper respiratory tract of patients not necessarily suffering from influenza, and (2) what variations in growth requirements exist among strains of these bacilli obtained from such sources. The results of this investigation are dealt with in the present paper.

Methods and Materials.

Autoclaved meat infusion broth was used as a basic medium. Filter-sterilized yeast extract, prepared in the manner described by Avery and Thjötta, supplied the V factor. 0.5 cc. of the extract was added to 5 cc. of broth. A solution of hematin, prepared in the manner described previously (11), supplied the X factor. Dilutions, 1 to 10,000, of this solution gave positive reactions for the presence of peroxidase. When the presence of the X factor was desired in the medium, 1 cc. of the hematin solution was added to each 100 cc. of broth. The broth or yeast extract alone never gave positive peroxidase tests.

Bacilli used in this work were obtained from the throats of patients by means of cultures on fresh 2 per cent rabbit blood agar plates. Hemolytic and non-hemolytic Gram-negative bacilli isolated from these plates were carried in stock on blood agar slants. A strain from the spinal fluid of a patient with influenzal meningitis and 1 from a pneumonic lung were also studied.

The bacilli to be tested were removed from blood agar slants by means of a platinum loop and transferred to tubes of broth containing yeast extract, hematin, and yeast extract and hematin respectively. In the case of non-hemolytic strains that grew only in broth containing both factors (X and V), and of hemolytic strains that required the addition of only the V factor, macroscopic evidence of growth was considered sufficient and no subcultures on solid media were made. Other strains, however, were tested more completely in regard to their growth requirements.

EXPERIMENTAL.

From 46 patients, 34 strains of so called hemophilic bacilli were isolated. In Table I the sources from which they were obtained and

the frequency with which they occurred in certain groups of cases are indicated. In some of the groups too few cases were studied to permit of a comparison between the groups. Omitting the 4 cases of influenza, however, 42 cases of other diseases were examined and the results are interesting and significant. From these 42 cases, 18 strains of non-hemolytic bacilli (43 per cent) and 11 strains of hemolytic ones (26 per cent) were obtained. Although the patients did not have influenza, 29 of them (69 per cent) harbored hemophilic bacilli in their throats. These findings are in accord with those of other workers who have shown that hemophilic bacilli are likely to be encountered very frequently during periods of epidemic respiratory infections.

TABLE I.

Sources of the Hemophilic Bacilli Studied.

Type of case	Number examined	Number of non-hemolytic strains isolated	Number of hemolytic strains isolated
Chicken-pox	27	8	7
Measles	9	6	3
Influenza	4	1	4
Miscellaneous	6	4	1
Total.....	46	19	15

The 34 strains of bacilli were tested in the manner described above to determine their need of growth accessory factors, V and X. The results are summarized in Table II.

From Table II it is obvious that 17 of the non-hemolytic strains required both growth accessory factors (X and V). They were similar in every respect to organisms usually accepted as influenza bacilli (Pfeiffer). There were 2 non-hemolytic strains, however, that grew well when yeast extract (V) alone was added to the broth. These organisms were carried through 7 successive transplants in this medium. Subcultures were pure. In broth alone or in broth plus hematin, with 0.25 cc. amounts for transfers, no viable bacilli were found by means of subcultures on blood agar after the 2nd transfer. Neither strain produced indole. Both reduced nitrates to nitrites.

Each grew diffusely in liquid media. These bacilli corresponded to Rivers' *B. parainfluenzæ.*

There were 10 hemolytic strains that grew well in broth to which only yeast extract (V) was added. These bacilli were fairly uniform in type. Cultures of them in liquid media usually exhibited flocculi similar to the ones seen in streptococcus cultures. On blood agar plates, colonies of the bacilli were surrounded by large zones of hemolysed red blood cells, were slightly opaque, and were often firm enough to be pushed about intact on the surface of the medium.

TABLE II.

Summary of Results Obtained Concerning the Growth Requirements of the Hemophilic Bacilli.

Type and number of strains	Broth + yeast extract	Broth + hematin	Broth + yeast extract + hematin	2 per cent rabbit blood agar
17 non-hemolytic	−	−	+	+
2 non-hemolytic	+	−	+	+
10 hemolytic	+	−	+	+
3 hemolytic	−	−	+	+
2 hemolytic	−	−	−	+
1 *B. influenzæ*, control	−	−	+	+
1 *B. parainfluenzæ*, control (original Rivers strain)	+	−	+	+
1 *B. hemoglobinophilus canis*, control	−	+	+	+

The + sign indicates visible growth. The − sign indicates no growth. Yeast extract supplied the V factor; hematin, the X factor.

Upon microscopic examination the bacilli were found to be exceedingly pleomorphic and usually larger than Pfeiffer's bacillus. In order to maintain stock cultures on blood agar slants it was necessary to make transfers more frequently than at weekly intervals.

Three hemolytic strains required both X and V growth accessory factors. They were carried through 7 successive transplants in broth containing yeast extract and hematin. In the later transfers, successful transplants were obtained by means of a platinum loop. In broth containing yeast extract or hematin alone no viable bacilli were present after the 3rd or 4th transfer. 2 other hemolytic strains that grew

well in rabbit blood broth were not viable after 3 or 4 transfers in broth containing both yeast extract and hematin. It is possible, however, that after a longer period of cultivation on artificial media, results with these 2 strains might have been similar to those obtained with the 3 strains described above. On blood agar plates colonies of these 5 strains were surrounded by very narrow zones of hemolysed red blood cells. They were small, transparent, moist, and soft. In fluid media the bacilli always grew diffusely. All 5 strains reduced nitrates to nitrites. One produced indole. Upon microscopic examination these organisms resembled Pfeiffer's bacillus. Weekly transplants of the organisms on blood agar were found to be sufficient for maintenance of stock cultures.

From the results of the experiments already described, it is evident that some hemophilic bacilli hemolyse red blood cells, while others do not, that some require both X and V growth accessory factors, while others require either X alone or only V. Fildes showed that organisms requiring only V, or both X and V, multiplied in symbiosis with *B. hemoglobinophilus canis* on media containing only X. From this observation he concluded that the latter organism synthesized V and supplied it to the bacilli needing it for growth. Since some hemophilic bacilli require the addition of only V to the media used for their cultivation, and since it has been shown that many bacteria capable of growing on ordinary media, *e.g.* staphylococci, give a positive peroxidase reaction, experiments were performed to determine (1) if the hemophilic bacilli capable of growing on media to which only V is added give a positive peroxidase test, and (2) if they are able to provide the X factor to bacteria needing it for growth. The results of these experiments will be described below.

In the experiments to determine whether the bacilli requiring the addition of only V to the media give a positive test for peroxidase, the following strains were employed: Rivers' original *B. parainfluenzæ*, 2 non-hemolytic strains which were described above and which will be spoken of as *B. parainfluenzæ* Nos. 2 and 3, and 1 hemolytic strain. The 4 strains of bacilli were grown in broth to which yeast extract had been added. Cultures 24 hours old were used in every instance. A portion of each culture was saved for tests. The remaining portion was centrifuged and the sedimented bacilli were resuspended in salt solution. This procedure was repeated twice. A portion of the original culture, the last supernatant salt solution, and the washed bacilli suspended in salt solution were

tested for the presence of peroxidase in the usual way by means of benzidine. The suspension of bacilli was much denser in the salt solution than in the original broth cultures. The results of the experiments are shown in Table III.

The results of the experiments summarized in Table III indicate that the hemophilic bacilli requiring the addition of only yeast extract (V) to the media give a positive test for peroxidase (X) in spite of the fact that the medium in which they are grown gives a negative one.

B. hemoglobinophilus canis requires the addition of X to its media, yet apparently produces V. *B. parainfluenzæ* and some of the hemolytic bacilli require the addition of V to their media, but give a positive test for peroxidase (X). In view of these facts it seemed of interest to ascertain whether the organisms requiring the addition of X alone and the ones needing the addition of only V are capable of supplementing the growth accessory factors for each other when seeded together in media to which neither X nor V has been added. The manner in which this question was investigated is described below.

TABLE III.

Results of Benzidine Tests for Presence of Peroxidase.

Strains tested	Original culture	Supernatant salt solution	Washed bacilli
B. parainfluenzæ, No. 1	±	−	+
B. parainfluenzæ, No. 2	−	−	±
B. parainfluenzæ, No. 3	−	−	±
Hemolytic bacillus	−	−	±

The ± sign indicates a faintly positive reaction. The + sign indicates a positive reaction. The − sign indicates no reaction.

Autoclaved meat infusion broth and meat infusion agar to which no growth accessory factors had been added were the media employed. *B. hemoglobinophilus canis* requiring the addition of X alone, *B. parainfluenzæ*, Nos. 1, 2, and 3, needing the addition of only V, and a hemolytic bacillus also requiring the addition of only V were the organisms studied. When a liquid medium was used transfers were effected by means of pipettes, 0.25 cc. being the size of the inoculum. Cultures were incubated 72 hours between transplants. Subcultures on blood agar plates were employed to determine the viability and the types of the bacilli in the different tubes. The ability of the bacilli to grow alone or together under the conditions mentioned is indicated in Tables IV and V.

TABLE IV.

Summary of Results of Experiments Concerning Symbiosis of Hemophilic Bacilli in Meat Infusion Broth.

Type and combination of bacilli	Factor supplied by each bacillus or combination of bacilli	Successive transfers						
		1	2	3	4	5	6	7
1. *B. hemoglobinophilus canis*	V	+	?	−				
2. *B. parainfluenzæ*, No. 1	X	+	?	?	−			
3. *B. parainfluenzæ*, No. 2	X	+	−					
4. *B. parainfluenzæ*, No. 3	X	+	?	−				
5. Hemolytic bacillus	X	+	?	−				
6. 1 and 2	V and X	+	+	+	+	+	+	+
7. 1 and 3	V and X	+	+	+	+			
8. 1 and 4	V and X	+	+	+	+	+		
9. 1 and 5	V and X	+	+	+	+	+	+	+

The − sign indicates no growth when subcultures were made on blood agar. The + sign indicates visible growth of bacilli of the type or types with which the series was started.

TABLE V.

Summary of Results of Experiments Concerning Symbiosis of Hemophilic Bacilli on Meat Infusion Agar.

Type and combination of bacilli	Factor supplied by each bacillus or combination of bacilli	Successive transfers						
		1	2	3	4	5	6	7
1. *B. hemoglobinophilus canis*	V	?	−					
2. *B. parainfluenzæ*, No. 1	X	?	−					
3. *B. parainfluenzæ*, No. 2	X	?	−					
4. *B. parainfluenzæ*, No. 3	X	?	−					
5. Hemolytic bacillus	X	?	?	−				
6. 1 and 2	V and X	+	+	+	+	+	+	+
7. 1 and 3	V and X	+	+	+	+			
8. 1 and 4	V and X	+	+	+	+	+		
9. 1 and 5	V and X	+	+	+	+	+	+	+

The − sign indicates no growth when subcultures were made on blood agar. The + sign indicates a visible growth of bacilli of the type or types with which the series was started.

From the results shown in Tables IV and V, it is obvious that bacilli requiring the addition of X alone and those requiring the addition of only V to their media grow well in symbiosis on media to which neither X nor V has been added. This fact indicates that these organisms are capable of supplementing the growth accessory factors for each other when grown together under the conditions of the experiments.

DISCUSSION.

The results of experiments reported in the first part of the paper support the findings previously recorded by Rivers and Fildes concerning the growth requirements of non-hemolytic and hemolytic hemophilic bacilli. Furthermore, these results indicate that a subdivision of this group of bacilli based upon their growth requirements is not only possible but also practicable. The majority of the non-hemolytic strains require the addition of both X and V to their media, while a few need the addition of only V. On the other hand, most hemolytic strains require the addition of only V to their media, while some need the addition of both V and X. It is true that 2 hemolytic strains were encountered that were susceptible to cultivation in blood broth but not in broth containing yeast extract and hematin. In view, however, of their recent isolation and of the difficulty experienced with 3 similar strains that required both factors, it seems justifiable to consider the 5 strains as members of one group, which, in its general characteristics, is more closely related to Pfeiffer's bacillus than to the other hemolytic bacilli under discussion.

The results of the investigation reported in the latter part of the paper apparently indicate that strains of hemophilic bacilli requiring the addition of only V or X to their media are capable of supplying, or acting as, X or V respectively to an extent sufficient for the needs of other bacilli that require the addition of these factors to their media. This fact supports the view that organisms which do not require the addition of either growth accessory factor to their media only multiply on such media because they are capable of synthesizing these factors.

Discussions have arisen in regard to the number of bacilli that should be included in the hemophilic group. Some workers contend

that this group of organisms should not be regarded as hemophilic, inasmuch as all of its members under certain conditions are capable of growth in the absence of blood. There is evidence, also, that in the past similar organisms have been classified under different headings, *e.g.*, Koch-Weeks' bacillus and Pfeiffer's bacillus are identical. Because of the variety of organisms that have been placed in this group any kind of a classification will have some objectionable features. Knowledge, however, recently acquired concerning the growth requirements of the hemophilic bacilli and the ability of some of them to hemolyse red blood cells enables one to outline a relatively simple classification of this heterogeneous group of organisms. Such a classification is presented below. The growth requirements, X and V, of a few bacilli placed in the hemophilic group have not been determined. When this has been accomplished these organisms can then be given their proper place in the classification presented.

Classification of Hemophilic Bacilli.

A. Requiring the addition of V and X to the media.
   B. influenzæ (*Hemophilus influenzæ*).
   1. Non-hemolytic.
   2. Hemolytic.
B. Requiring the addition of only V to the media.
   B. parainfluenzæ (*Hemophilus parainfluenzæ*).
   1. Non-hemolytic.
   2. Hemolytic.
C. Requiring the addition of only X to the media.
   B. hemoglobinophilus canis (*Hemophilus canis*).
D. Requiring the addition of neither X nor V to the media.
   1. *B. pertussis* (*Hemophilus pertussis*).

SUMMARY.

1. 19 strains of non-hemolytic hemophilic bacilli were studied. 17 required the addition of V and X growth accessory factors to their media, 2 required the addition of only V.

2. Of 15 strains of hemolytic hemophilic bacilli examined, 10 were found to require the addition of only V to their media, 3 the addition of V and X in the form of yeast extract and hematin, and 2 the addition of accessory growth factors in the form of blood.

3. In media to which neither V nor X had been added true symbiosis was found to occur on growing *B. hemoglobinophilus canis*, requiring the addition of X, with *B. parainfluenzæ*, requiring the addition of V, or with hemolytic strains of bacilli, requiring the addition of only V.

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A SIMPLE METHOD FOR THE ISOLATION OF PURE CULTURES FROM SINGLE BACTERIAL CELLS.

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The use of "pure line" strains of bacteria is required in many problems encountered in bacteriological research. A number of methods have been devised for obtaining cultures which represent the progeny derived from a single cell. Most of these methods possess at least one of the following disadvantages: (1) intricate apparatus, (2) complicated technique, (3) loss of time, due to the frequent failure of a single cell to grow when transplanted.

During studies on variants occurring in streptococcus cultures, a simple method was devised by which pure line strains can be obtained with comparative ease. This method represents a modification of the Hansen[1] method for the isolation of pure cultures of yeast. Although Hansen's method has been employed extensively in the fermentation industries for a number of years, its most advantageous features have not been incorporated in any of the previous bacteriological methods. The essential features of the method as adapted to the isolation of single cell cultures of bacteria are described below.

Apparatus and Materials.

Cover-Glass.—A large 43 × 50 mm. cover-glass, 0.13 mm. thick, is ruled into squares of 4 sq. mm. area by etching with a fine needle and hydrofluoric acid through paraffin. The system of numbering the squares is illustrated in Fig. 1. *Ring.*—The cover-glass is supported on a metal ring. Convenient dimensions proved to be 35 mm. diameter, 3 mm. height and 1 mm. thickness. The ring can be cut

[1] Hansen, E. C., *Meddelelser Carlsberg Lab.*, 1886, ii, No. 4, 152.

from brass tubing. The metal side arms are attached for convenience in handling. *Glass Slide.*—The chamber is formed by attaching the ring to a large slide 50 × 75 × 1 mm. The assembled apparatus is shown in Fig. 2.

Inoculating Spatula.—The spatula used for inoculation is formed from a glass rod 4 mm. in diameter. One end is drawn out in a flame to 2 mm. in diameter, and the tip flattened and bent at a convenient angle.

Culture Medium.—Any clear solid medium suitable for the cultivation of the test organism may be used provided it is made as free as possible from all extraneous particulate matter. Freedom from particles in the culture medium is essential to the success of the method.

Technique.

All glassware should be scrupulously clean before use. Rings, glass slides and cover-slips are sterilized in separate Petri dishes by dry heat. Aseptic precautions are followed throughout the technique. The assembling of the different parts is done under the protection of the lifted Petri dish cover. The metal ring is dipped in hot paraffin and then placed in contact with the unetched side of the cover-slip. The paraffin is allowed to harden. The surface of the cover-glass within the ring is flooded with melted agar sufficient to cover the entire area. In order that the film formed may be the thinnest possible, a warm pipette is held at right angles to the surface and all the recoverable agar is withdrawn and discarded. After this delicate film of agar has hardened it is inoculated as follows: A loopful of a young broth culture is transferred to 5 cc. of infusion broth and thoroughly mixed. The sterile glass spatula is dipped into the dilute bacterial suspension and the excess drained off against the side of the tube. The spatula carrying the minimum inoculum is lightly stroked over the entire surface of the film. The inoculated cover-glass and attached ring are inverted and sealed to the large glass slide which has been previously ringed with paraffin.

The preparation is then examined under the microscope with a high dry objective. After a thorough search, squares containing only one organism are selected and the location of each plotted on a diagram with reference to the index number. The location of the

cell within the selected square is also recorded on the diagram. The orientation is further facilitated by the use of a mechanical stage. Usually six or seven squares, each containing a single organism, are plotted to insure against any failure of growth.

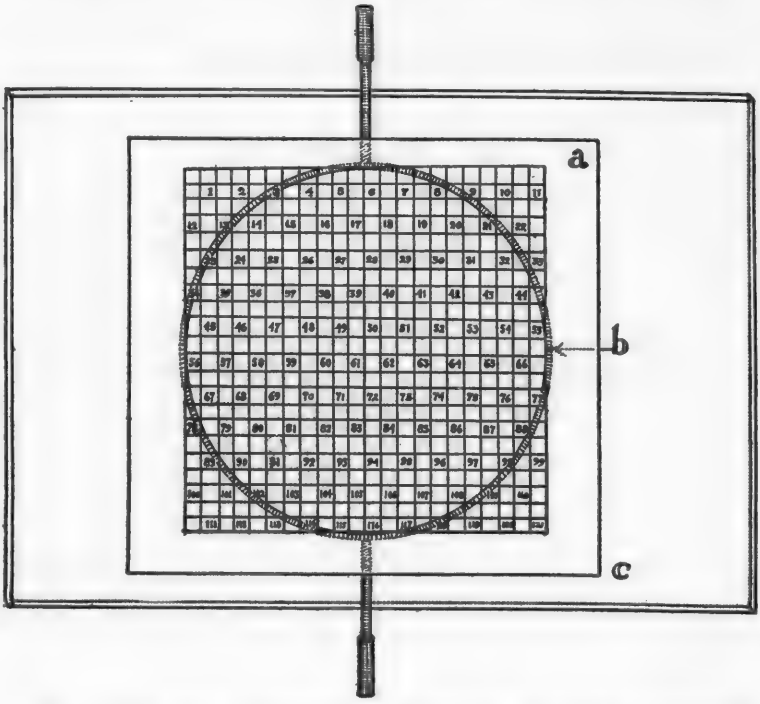

FIG. 1. Top view of apparatus. (*a*) Cover-glass; note that all squares not numbered are adjacent to numbered ones. (*b*) Brass ring and side arms. (*c*) Glass slide.

The entire apparatus can now be removed from the microscope stage and incubated at 37°C. until colony formation is well developed. At the end of the incubation period the length of which is determined by the growth rate of the particular organism, the preparation is again examined microscopically. With the aid of the diagram and the stage readings, the fields previously selected are located and the

individual square is again searched to make certain that it contains only a single colony. As each individual square is checked, its position on the cover-glass is indicated by an ink dot made with a fine pointed pen while the preparation is under low power magnification. The ring carrying the cover-glass is then removed and by means of a fixed reading lens the colony indicated by the dot is transferred with a fine platinum needle to a suitable culture medium.

The principal sources of error in the method are practically eliminated by the selection of cover-glasses of the proper thickness and by the use of a culture medium which is wholly translucent and free as possible from all microscopic particles. If the agar is spread in a very thin film, little difficulty is experienced in the selection of single bacterial cells, when the illumination is adjusted. The moisture of

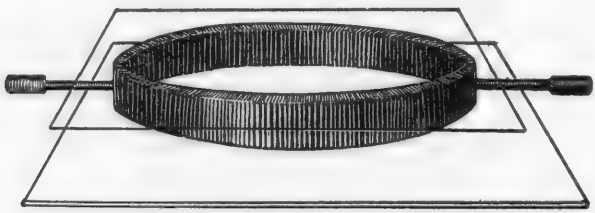

FIG. 2. Side view of apparatus.

the shallow chamber is maintained by the agar medium, which makes it possible to omit the excess fluid usually added in moist chamber preparations The excess moisture is not only unnecessary but is undesirable since the coalescence of drops of condensed water befog the field of vision and tend to cause the migration of bacterial cells.

COMMENT.

The procedure as outlined has been used in the study of streptococci and pneumococci but it is equally adapted to the isolation of other aerobic organisms and by slight modification might be made useful in the study of anaerobic types.

The method is simple in operation and no special technique is required to obtain the results desired. That several selected colonies can be isolated from the same preparation is most advantageous,

since the chance of successful transplant is thereby correspondingly increased. Moreover, the fact that a number of different colonies can be kept under observation on the same preparation, makes the method particularly adaptable to studies on variation in colony structure. In a successful preparation, it is often possible to obtain a dozen colonies, each of which is known to be derived from a single cell. This is a distinct advantage in the study of the origin of variants which differ in colony structure.

SUMMARY.

A method is described for the isolation of pure cultures from single bacterial cells, which is simple in operation and which requires no complicated apparatus. In addition to its simplicity, the method possesses the further advantage that several selected colonies can be isolated from the same preparation.

THE CULTIVATION OF LENS EPITHELIUM IN VITRO.*

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PLATE 30.

(Received for publication, March 14, 1927.)

In the course of an investigation into the nature of the nutrition of the crystalline lens and the cause of cataract, it was proposed by the author that an attempt be made to cultivate the cells of the lens *in vitro*. Such a strain of tissue might be used for controlled experiments.

Since the discovery by Harrison (1) in 1907 that cells could be caused to migrate and divide outside the body, various investigators have used the method for the study of morphological and physiological problems.

Carrel (2) developed an ingenious technique by which permanent pure strains of fibroblasts could be isolated and cultivated *in vitro*. He established the procedures by which the method of tissue cultivation is becoming adapted to its main object, the study of the fundamental problems of physiology and pathology. The early technique consisting of placing a fragment of tissue in a hanging drop of culture medium, did not allow an accurate analysis of the action of a tissue upon other tissues and upon the humors. The cells were subjected to complex influences, such as those of necrotic cells of their own type, of living and dead cells of other types, and of a medium which deteriorated spontaneously in a short time (3).

Advances have been made during the last few years, chiefly through the embryonic extract-plasma clot method of Carrel by which those difficulties and

* The basis of this report is derived from work conducted by the author as Research Fellow for the American Academy of Ophthalmology and Oto-Laryngology and by virtue of a grant secured from an anonymous donor by Dr. John M. Wheeler. The experiments were carried on in the Departments of Anatomy, Physiology, and Chemistry at the University and Bellevue Hospital Medical College. Acknowledgements are gratefully made.

objections are overcome, and have contributed markedly to the progress of an important method of investigation, primarily by the isolation of pure strains of cells (4), and by the development of procedures for measuring the rate of growth and for studying the functional activity of the tissues (5, 6).

Fischer (7) isolated an unmixed strain of epithelium from the anlage of the chick iris in 1922. Ebeling (8) produced cultures of the thyroid epithelium in 1925. It has been possible to carry these cultures on for long periods of time. It has been proved that the cells remain true to type, that they do not dedifferentiate, and that they produce their typical substances *in vitro*.

In the experiments about to be reported the method and technique of Carrel (2–6), were used.[1]

Experiment Demonstrating the Metamorphosis of the Lens in Vitro.

The eyes of two chick embryos of about 52 hours incubation (lens vesicle stage) were removed and the anterior third of each eye placed in a homogeneous medium consisting of equal parts of adult chicken plasma and chick embryonic extract. The concave slide method of Carrel was used. The explant was oriented so that the lens vesicle in each could be plainly observed with the microscope. They were allowed to incubate for 72 hours at 37.5°C. and in two of the specimens it was observed that differentiation of the posterior portion of the lens vesicle had occurred, the cells having elongated to form lens cortex. In the other two, due to digestion of the medium, the specimens were not in proper position for observation. Microscopic sections of the specimens were not obtained. Since this work, the author has learned of the explants of chick embryo eyes by Strangeways and Fell (9). The latter workers demonstrated by microscopic sections the differentiation of the eyes which progressed *in vitro* in a surprisingly normal way.

The Cultivation of the Crystalline Lens Epithelium in Vitro.

Experiment I.—The lens and vitreous of chick embryos of from 4 to 9 days incubation at 37.5°C. were removed by incising the posterior portion of the optic vesicle and using traction on the vitreous. Usually a ring of pigmented and non-pigmented epithelium of the rim of the optic vesicle comes away with the lens and vitreous. If the vitreous be excised and the remaining explant cultivated in the manner used by Fischer (7) a growth of epithelium from the iris anlage is obtained.

This experiment demonstrates that the epithelium of the iris anlage can be cultivated *in vitro* and also that if an unmixed strain of lens

[1] Acknowledgements are gratefully made to Drs. Alexis Carrel and Albert H. Ebeling for their many courtesies.

epithelium is desired, all extraneous tissue must be removed from the lens. Strains of iris epithelium were carried along in successive subcultures, so that a ready comparison with the cultures of the lens epithelium was always available.

Experiment II.—After many experiments, it was found that the eyes of chick embryos incubated for 5 days at 37.5°C. were ideal for dissecting the crystalline lens free from all extraneous tissue. The eyes usually measured 3 mm. in diameter and the lens slightly less than 1 mm. in equatorial diameter. Both prior to this stage and after it, the protoplasmic adhesions are such as to make it almost impossible to express the lens free from other cells. The embryo is placed in a concave slide and the amnion removed from the eye region. A sharp, cutting needle in a holder is used in each hand, one to steady the eye and the other to incise the posterior portion of the eye and draw out the vitreous on the point of the needle, bringing with it the lens and a portion of the rim of the optic cup. This specimen is placed in Tyrode (10) solution in a clean concave slide, under the binocular dissecting microscope using oculars 2 objectives A2 with transmitted light. The needles are replaced by fresh ones. These are inserted between the equator of the lens and the investing tissue, the other needle gently expresses the lens free from all extraneous tissue. This is transferred to fresh Tyrode solution and then to the embryonic extract-plasma clot medium. After 72 hours incubation it was observed that no outgrowth of cells had occurred.

This experiment demonstrates that it is possible to dissect out the crystalline lens of a chick embryo of 5 days incubation in such a manner as to free it from all extraneous tissue, and that the lens epithelium is contained within a cuticula outside of which when uninjured the cells do not migrate. (Salzmann (11) found that the lens vesicle is soon closed off from the neighboring structures by a cuticula, later the lens capsule.)

Experiment III.—Lenses from 5 day chick embryos isolated in the manner described in Experiment II were incubated *in toto* for 24 hours at 37.5°C. The cultures were then opened and the lenses removed from the media and divided into two pieces. Outgrowths of cells were obtained from the epithelium of two of four lens incubated. One of these, No. 667–2, represented the beginning of a strain of lens epithelium which will be described in Experiment VI as Strain C.

Those incubated for a longer period apparently retained no viable cells as no outgrowths of cells were obtained after section. Some of these specimens took up and retained the neutral red vital stain (1–30,000) during the first period of incubation. The trauma of the expression of these particular lenses may have had something to do with their inability to withstand cultivation outside the

body. Undoubtedly the cuticula of this stage (later the lens capsule) has much to do as a semipermeable membrane with the process of nutrition of the embryonic lens and a slight injury to this membrane would destroy the possibility of its living *in vitro*.

24 hours is sufficient time for incubation of tissue from a 5 day embryo to exclude any growth of cells extraneous to the cuticula of the lens. In our experiments on tissue from this age embryo, growth if it is to occur, will be evident after 24 hours.

This experiment demonstrates that it is possible to rule out tissue extraneous to the cuticula of the embryonic lens by cultivation and then by section of the lens to obtain a culture of an unmixed primary culture of epithelium from the interior of the embryonic lens.

Cultivation of Lens Epithelium in Vitro.

Experiment IV.—Lenses of 5 day embryos were expressed on numerous occasions in the manner described in Experiment II. Having been placed in fresh Tyrode solution, and again replacing the needles for fresh ones, the lenses were incised anteriorly and the lens epithelium stripped off easily to the equatorial region, as there exists a potential space at this stage between the lens epithelium and the cortex. The lens epithelium is then explanted on the point of a needle directly to the embryonic tissue juice-plasma clot medium. The cortex is explanted into another slide. Incubated at 37.5°C.

After 12 to 24 hours the lens epithelium begins to migrate, divide by mitosis, and multiply. After 48 hours observations are recorded. Apparently the entire explant lives, but cells do not wander out from all parts. This depends on the particular conditions of the explant, foldings, the exposure of the cells, the trauma, and the conditions of the media. Many bizarre shapes have been observed. Small pieces of epithelium do as well as large ones. Cells apparently temporarily united move out from the explant, never losing contact, however. The walls of cells in apposition are difficult of definition in the living cultures, but apparently the cells do not form a syncytium. Both membrane and peninsula formation are observed. The explant remains transparent and the newly formed cells are transparent. The wall of the healthy cell is a geometric line. Protoplasmic processes of various lengths are seen, some being very long. The cytoplasm is clear, containing granules some of which take up neutral red in weak solution and others which do not. The mitochondria are visible in the unstained cells. When the microscope substage diaphragm is opened and slight excess light admitted, the cells become invisible. The nucleus is ovoid and usually contains one or two nucleoli. All stages of mitoses may be observed. About the entire living culture there is observed a halo of transparency of the media, indicating either, the influences of the cell products on the media or, the preparation of the media by the

cells for their nutriment. The outgrowth of the cells is apparently preceded by a process of digestion of the media.

Fresh explants of lens epithelium should be cultivated on No. 0 glass cover-slip and examined with the aid of high magnification to prove the absence of any contaminating cells. Fibroblasts can be differentiated and the living pigment epithelial cells of the eye if present will show definite completely opaque rod-shaped ovoid or rounded pigment granules. The nature of these cells from the iris anlage and their appearance when cultivated *in vitro* must be thoroughly studied by anyone attempting to cultivate lens epithelium.

The explants of cortex do not grow. No cells wander or divide. Partial solution of the explant takes place by a process of hydrolysis. Disintegration of the cells occurs and the explant has a fibrillated, washed out appearance. This process can be best observed by allowing the cortex to remain in Tyrode solution and observing it with the microscope. Hydration and swelling will occur quickly, a beaded appearance is produced by globules forming in the cells. These globules rupture, the cell bursts, and the cytoplasm goes out into the solution which is isotonic for other tissues but hypotonic for lens cortex. A solution containing 1.2 per cent NaCl is isotonic for the lens cortex in that this hydrolysis does not take place, but cell migration and division do not occur. The cells are, even at this early stage of differentiation, biologically old. They have a different osmotic coefficient than the anterior lens epithelial cells.

This experiment demonstrates that lens epithelium when cultivated *in vitro* under proper conditions will migrate, divide, and multiply, and that under these same conditions, the lens cortex cells will not do so. A distinction is drawn between lens epithelium which is limited to the anterior and equatorial regions of the lens and the lens cortex which constitutes the differentiated bulk of the lens. They are both epithelial. From the anatomic arrangement of the cells the anterior epithelium and their elongation at the equatorial region passing over into the lens cortex, it may be safe to assume that the cells of the cortex are derived from the epithelial layer, or at least from those cells which are at the equator. It may be possible to grow the cells of the cortex, as long as they retain their nuclei, under different conditions of cultivation. Alterations have been made in the media, but no successful cultures have been obtained. These facts if substantiated by further experiment would lend proof to the idea that the anterior epithelium of the lens differentiates into the cortex.

Experiment V.—Extract of the 9 day chick embryo was used in place of extract of the whole body. It was made by grinding the eyes in a Wedgwood mortar

with pestle and alkali-free ground glass. It was diluted with Tyrode solution and centrifuged. The explants grew well when this was used in place of the whole body extract, but were not free from retinal pigment rods in the cells. Now either pigment cells had been explanted with the lens, or the cells were transferred with the eye extract, or the pigment rods in the eye extract were ingested by the cells. It has already been demonstrated that the lens can be explanted free from pigment rods and these could be seen with the aid of oil immersion lens. It was therefore determined to use extract of the embryo body minus the eyes. This has been done and transparent growths free from any retina pigment rods obtained. Dr. Albert H. Ebeling has been kind enough to examine some of our cultures and has found no contaminating cells in one produced by our perfected technique.

This experiment demonstrates that lens epithelium can be cultivated in media containing extract from the embryonic eye alone and also from the embryonic body minus the eyes. In the latter media, the cells can be proved free from any contaminating cells.

Experiment VI.—To produce an unmixed strain of lens epithelium which can be carried along indefinitely and used for controlled experiments.

Living explants from lenses isolated and cultivated as described in Experiment IV can be divided and two subcultures obtained. These can in turn be subdivided after cultivation and thus secondary and tertiary cultures obtained. By this means a strain of lens epithelium can be propagated from an original single explant. One of our strain (C) was obtained as described in Experiment III after having incubated the entire lens proving the absence of any cells extraneous to the cuticula. The history of this strain may be reported as exemplifying the culture of lens epithelium under these conditions.

June 28, 1926 (No. 667–3). Entire lens explanted from a 5 day embryo.

June 29 (No. 669–2). The lens remains transparent. Dissected out of the media. Cortex shelled out. Found granular, partially disintegrated. Epithelium subcultivated.

July 1. Several processes. Cellular outgrowth from explant.

July 3. Small growth, vacuolated cells. Media changed.

July 6. Good growth of cells. Membrane and peninsula formation.

July 7, 9, 12, 14, 16, 19, 21, 23, 26, 28, 30, Aug. 2, 4, 6. Media changed and culture divided and subcultivated.

One-half of the culture was put on mica cover-slip and one-half on glass cover-slip. Latter examined on the ensuing working day with oil immersion lens and then fixed and stained for further study. The culture on mica was used for the propagation of the strain. Extract of the embryonic body minus eyes was used throughout. The divided cultures about doubled in size every 48 hours. The cells are transparent and remain epithelial in type. They do not dedifferentiate.

This experiment demonstrates that lens epithelium cultivated *in vitro* can be carried on for successive generations. Strain C was carried through seventeen generations over a period of 6 weeks and was thriving when the work was discontinued, August 9. It was then ready for controlled experiments and observations on the rate and character of the growth, the cellular reactions, and the substances produced by the cells.

CONCLUSIONS.

1. Differentiation of the posterior cells of the lens vesicle into lens cortex has been observed *in vitro*.
2. It is possible to dissect out the lens of the 5 day embryo chick in such a manner as to free it from all extraneous cells.
3. Lens epithelium even at a very early stage is contained within a cuticula.
4. Lens epithelium under proper conditions will live, migrate, divide, and multiply *in vitro* in primary explants.
5. An unmixed strain of lens epithelium can be propagated from a primary explant by successive subcultures. This strain can be utilized for controlled experiments on the nutrition of the cells of the lens.

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EXPLANATION OF PLATE 30.

FIG. 1. Fresh explant of lens epithelium. Zenker acetic-Harris hematoxylin. × about 175.

FIG. 2. Showing mitosis and membrane formation in a strain of lens epithelium. × about 355.

FIG. 3. Cells from tip of peninsula in a strain of lens epithelium showing fine protoplasmic processes. × about 495.

FIG. 4. Cells from tip of peninsula in a strain of lens epithelium. Herniations of cytoplasm as first stage of degeneration of these cells. × about 495.

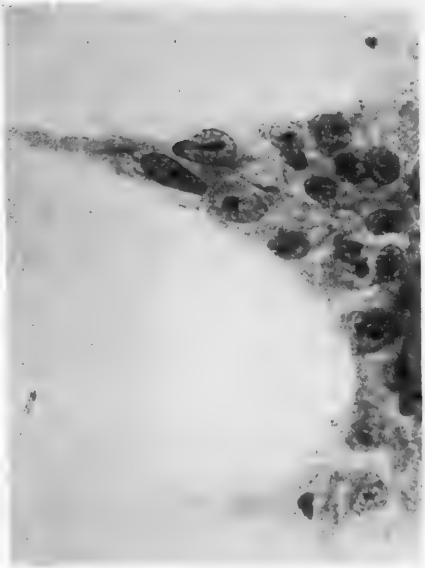

FIG. 1.

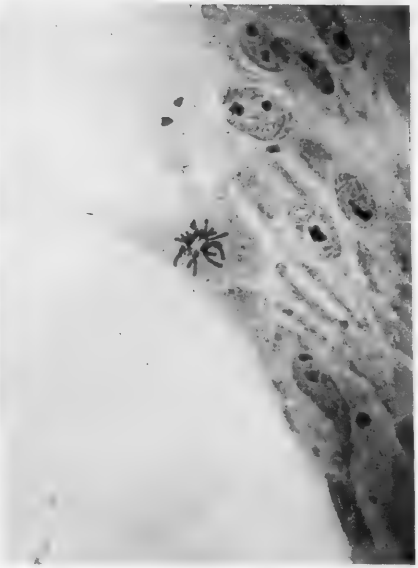

FIG. 2.

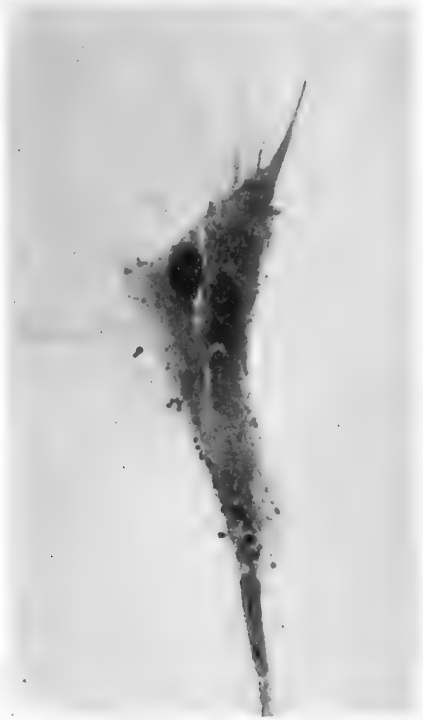

FIG. 3.

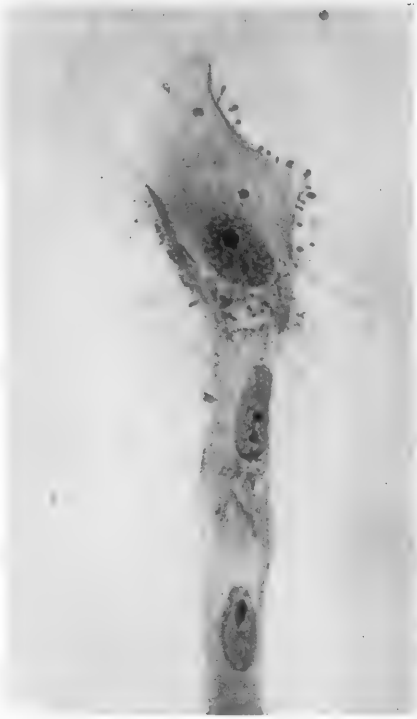

FIG. 4.

(Kirby: Cultivation of lens epithelium.)

THE ACTION OF THE SYMPATHETIC UPON THE EXCITATORY PROCESS IN THE MAMMALIAN HEART.

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The heart *in situ* is normally under the balanced control of its extrinsic nerves, preganglionic fibers of the parasympathetic system which enter *via* the vagus and end about ganglia in the cardiac tissue, and postganglionic fibers of the sympathetic group which are widely distributed to the muscular and specialized tissues.

The influence of the vagus upon the origin and propagation of the excitatory process has been studied in detail. In the mammalian heart it acts to slow the spontaneous rhythm whether this arise in the sinoauricular or auriculoventricular node. The sinus is chiefly under the control of the right nerve while the A-V node is governed by both (1). Vagus stimulation brings about slowing of A-V conduction, the left nerve being the more effective (2). It may also cause partial block in the bundle branches (3). Intraauricular conduction is retarded in the cold blooded heart by vagus stimulation (4). In the auricle of the dog the normal conduction is not altered but if the transmission has been previously slowed it is accelerated by the vagus (5). The refractory period of the auricular muscle is shortened (6) while that of the ventricle is unaffected (7).

While it appears that all portions of the heart are under the influence of the sympathetic its action is most effective at the normal centers of rhythm, the sinoauricular and auriculoventricular nodes (8). At these points there exists a noticeable difference between the influence of the right and left nerves, the right producing primarily an acceleration of the sinus rhythm while the left enhances A-V conduction (9, 10). And finally, when the sympathetic endings are stimulated by means of adrenalin, conduction within the ventricle is accelerated (11).

The present study was undertaken in an attempt to examine certain phases of the action of the sympathetic which have thus far escaped careful investigation.

Effect of Sympathetic Stimulation on Intraauricular Conduction.

The influence of adrenalin upon the transmission of the excitatory process in the auricle after functional exclusion of the vagus, has not to our knowledge been reported. We have, therefore, determined the intraauricular transmission rate before and after adrenalin in a series of eight experiments.

The animals were anesthetized with urethane and the hearts exposed *in situ*. Direct leads were taken from the right auricle by means of non-polarizable electrodes. Adrenalin in 1/50,000 dilution was injected into the femoral vein.

The results of three such observations are shown in Table I. Beginning within ½ to 1 minute after the intravenous injection of adrenalin there is a rise in transmission rate of from 100 to 400 mm. per second, varying with the amount of drug injected but occurring in some cases after doses too small to cause any considerable increase in the rate of the sinus rhythm.

Paralysis of the Sympathetic.

Dale (12) has prepared from ergot a substance, ergotoxin, which consistently depresses the functions of the sympathetic system. More recently Stoll (13) has isolated a pure alkaloid, ergotamine, having similar properties but in greater dilution. This substance antagonizes the pressor action of adrenalin upon the systemic arteries, and inhibits its dilator effect upon the coronaries (14). In dilution 1/100,000 it neutralizes the accelerating action upon the frog's heart of 1/2,000,000 adrenalin (15). It depresses the tendency of adrenalin to produce hyperglycemia in rabbits (16) and relieves the inhibitory action of that drug upon the gut (14). In short, ergotamine has a paralyzing effect upon the sympathetic endings similar to that exerted by atropine upon those of the vagus.

Ergotamine tartrate (0.25–1.0 mg.) was injected intravenously in cats and dogs and the heart rate recorded by galvanometric records.

TABLE I.

Effect of Adrenalin upon Intraauricular Transmission.
Normal Sinus Rhythm.

	Time	Rate	Transmission rate	Remarks
			mm. per sec.	
Experiment 4. Vagi excluded. Electrodes 20 mm. apart	2.35	216	1083	
	2.47	218	1056	
	2.48	210	1065	
	2.49½			0.25 cc. adrenalin 1/50,000
	2.50	220	1170	
	2.51	214	1160	
	2.55	214	1167	
	2.59	210	1121	
	3.02			0.5 cc. adrenalin 1/50,000
	3.02½	216	1153	
	3.03	206	1257	
	3.07	206	1145	
	3.12	206	1029	
	3.14			1.0 cc. adrenalin 1/50,000
	3.15	212	1397	
	3.19	202	1107	
	3.24	206	1100	
Experiment 6. Vagi excluded. Electrodes 20 mm. apart	2.43	179	892	
	2.45	169	865	
	2.47	170	841	
	2.48½			0.5 cc. adrenalin 1/50,000
	2.49	192	1245	
	2.49½	207	1260	
	2.50	200	1038	
	2.51½	178	957	
	2.53	172	915	
	2.58	173	835	
	3.04	170	861	
Experiment 7. Vagi excluded. Electrodes 20 mm. apart	2.58	194	803	
	3.00	185	875	
	3.10	183	723	
	3.12½			1.0 cc. adrenalin 1/50,000
	3.13	191	972	
	3.13½	192	1034	
	3.14	186	1005	
	3.16	182	936	
	3.18	179	897	
	3.23	171	819	
	3.28	169	806	

The animals were anesthetized with urethane and artificial respiration maintained throughout the observation.

In a series of five experiments carried out on animals with the vagi intact the observations of previous workers (14) were confirmed, namely that ergotamine slows the rate of beat and that this slowing is relieved by atropine.

Thus in the experiment outlined in Table II, the injection of 0.25 mg. ergotamine was followed by a slowing of the sinus rhythm from 222 to 136 per minute. A second dose soon after the first failed to

TABLE II.

Effect of Ergotamine upon Sinus Rhythm with Vagi Intact.
Experiment 5. Cat 1650 Gm.

Time	R-R	Rate	P-R	Remarks
3.48	.26	230	.07	
3.49	.27	222	.07	
3.50				0.25 mg. ergotamine
3.52	.40	150	.08	
3.54	.44	136	.08	
3.55	.40	150	.08	
3.56				0.25 mg. ergotamine
3.58	.44	136	.08	
4.00	.40	150	.09	
4.02				0.25 mg. atropine
4.04	.25	240	.10	
4.06	.25	240	.10	
4.10	.25	240	.10	

produce any pronounced change. After 0.25 mg. atropine, however, the rate rose again to 240.

Such results are obviously susceptible of two explanations: either that ergotamine stimulates the vagus, or that it acts to diminish the accelerating action of the sympathetic. In an attempt to analyze the effect more closely ergotamine was administered after the vagi had been functionally excluded. Both nerves were sectioned in the neck and atropine sulfate, 2.0 mg., was injected intravenously. Following this procedure ergotamine (0.5–1.0 mg.) still produced conspicuous slowing of the sinus rhythm and a prolongation of the P-R interval of the electrodardiogram as shown in Table III. Moreover,

TABLE III.

Effect of Ergotamine upon Sinus Rhythm with Vagi Functionally Excluded.

	Time	Rate	P-R	Remarks
Experiment 9. Dog 5.0 kilos. Vagi excluded	2.36	167	.09	
	2.39	167	.09	
	2.40			1.0 mg. ergotamine
	2.41	125	.12	
	2.42	125	.12	
	2.44	120	.12	
	2.46	118	.12	
	2.51	115	.12	
Experiment 10. Dog 6.2 kilos. Vagi excluded	3.10	260	.07	
	3.13	250	.07	
	3.14			0.5 mg. ergotamine
	3.15	200	.08	
	3.16	187	.11	
	3.19	187	.09	
	3.21			1.0 cc. adrenalin 1/10,000
	3.22	187	.08	
	3.23	182	.08	
	3.25	182	.10	
Experiment 12. Dog 7.4 kilos. Vagi excluded	12.10	240	.08	
	12.14	250	.08	
	12.15			0.5 mg. ergotamine
	12.16	200	.10	
	12.17	187	.10	
	12.19	172	.11	
	12.25	167	.11	
	12.28			1.0 cc. adrenalin 1/10,000
	12.29	177	.10	
	12.32	167	.12	
Experiment 13. Dog 6.5 kilos. Vagi excluded	2.10	222	.07	
	2.12	220	.07	
	2.14	222	.08	
	2.15			1.0 mg. ergotamine
	2.16	194	.10	
	2.17	188	.11	
	2.18	182	.10	
	2.19	182	.10	
	2.21	167	.10	

TABLE IV.

Effect of Ergotamine upon Intraauricular Transmission. Normal Sinus Rhythm and Rhythmically Driven Heart.

	Time	Rate	Transmission	Remarks
			mm. per sec.	
Experiment 26. Vagi excluded. Electrodes 21 mm. apart. Normal sinus rhythm	2.28	230	878	
	2.30	244	910	
	2.31			1.0 mg. ergotamine
	2.33	214	834	
	2.38	209	836	
	2.48	204	788	
	2.53	182	803	
	3.00	172	796	
Experiment 27. Vagi excluded. Electrodes 24 mm. apart. Normal sinus rhythm	2.37	135	922	
	2.40	139	955	
	2.42	136	949	
	2.43			1.0 mg. ergotamine
	2.47	119	918	
	2.51	123	885	
	3.10	135	809	
	3.40	128	847	
	4.10	122	817	
	4.40	121	733	
Experiment 19. Vagi excluded. Electrodes 24 mm. apart. Heart rhythmically driven	3.00	153	732	
	3.01	153	762	
	3.03	153	751	
	3.04			1.0 mg. ergotamine
	3.05	153	678	
	3.07	153	674	
	3.10	153	644	
	3.18	153	636	
	3.22	153	646	
Experiment 20. Vagi excluded. Electrodes 26 mm. apart. Heart rhythmically driven	3.23	214	863	
	3.24	214	816	
	3.26	214	877	
	3.27			1.0 mg. ergotamine
	3.28	214	699	
	3.29	214	624	
	3.34	214	635	
	3.38	214	620	
	3.43	214	623	

TABLE IV—*Concluded.*

	Time	Rate	Transmission	Remarks
			mm. per sec.	
Experiment 25. Vagi excluded. Electrodes 24 mm. apart. Heart rhythmically driven	2.47	216	970	
	2.50			1.0 mg. ergotamine
	2.55	216	1040	
	2.58	216	997	
	3.00	216	995	
	3.05	216	847	
	3.12	216	781	
	3.20	216	788	

the subsequent injection of adrenalin in doses of 1.0 cc. of a 1/10,000 solution failed to cause acceleration. Such results indicate that ergotamine may effect slowing of the sinus rhythm and cause delay in A-V conduction quite apart from any action upon the vagus. Finally a series of twelve observations was carried out to determine the action of ergotamine upon intraauricular conduction. The results in Table IV indicate that, whether the auricles are responding to the normal sinus rhythm or to rhythmic induction shocks, ergotamine brings about a slowing in the rate of transmission of the excitatory process of from 100 to 200 mm. per second. In the majority of instances this effect comes on gradually and reaches its maximum from 20 to 30 minutes after the injection.

DISCUSSION.

When the action of the parasympathetic nerves has been excluded by means of atropine it is possible to demonstrate that the excitatory process in the heart is affected to a considerable degree by the control of the sympathetic nerves. Upon all phases of excitation and conduction in the mammalian heart with the exception of intraauricular conduction the action of the vagus and sympathetic are opposed. Here the vagus is without effect unless the rate of transmission is abnormally slow. Under such circumstances it may bring about acceleration but never to a rate above the normal. Stimulation of the sympathetic nerves on the other hand invariably causes a conspicuous rise in the transmission rate. It is therefore apparent upon closer analysis

that the effects of the vagus and sympathetic nerves upon intraauricular conduction are in no sense synergistic.

Under normal conditions there exists a delicate balance of such a nature that stimulation of one system involves inhibition of the other. It is, however, significant that, following paralysis of both sympathetic and parasympathetic nerves, the normal rhythm is maintained spontaneously at a more or less constant rate.

SUMMARY AND CONCLUSIONS.

Stimulation of the sympathetic nerve endings, after paralysis of the vagus, accelerates the transmission of the excitatory process in the dog's auricle.

Following the functional exclusion of the vagus ergotamine slows the rate of the sinus rhythm, depresses A-V conduction, and delays the transmission of the excitatory process in the auricle.

The relation of the sympathetic and parasympathetic nerves in the control of the cardiac rhythm is discussed.

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EXPERIMENTAL EPIDEMIOLOGY OF TUBERCULOSIS.

The Elimination of Tubercle Bacilli in the Feces, Bile, and Urine of Infected Guinea Pigs.

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The study of an induced epidemic of spontaneous tuberculosis in guinea pigs, the results of which have been reported in a previous paper (1), has shown that the portal of entry of the infection is with few exceptions the intestinal tract, the mesenteric lymph nodes being secondarily involved. It has seemed desirable to determine whether the elimination of tubercle bacilli in the feces of experimentally infected guinea pigs is a source of infection and causes the spread of spontaneous tuberculosis.

It has been demonstrated that bacteria introduced into the circulation may be eliminated through the intestinal tract. Emmerich (2) in 1885 recovered the cholera vibrio from the intestinal tract of cats 6 hours after intravenous or subcutaneous inoculation. Hess (3) in 1910 found that *B. prodigiosus* introduced into the circulation is eliminated from the blood through the liver and the kidneys 1 hour after its injection and even after he tied off the duodenum just below the common bile duct and the canal of Wirsung he was able to recover the bacilli in the small intestine 2 hours after injection. Following the ligation of the common bile duct in guinea pigs, Ribadeau-Dumas and Harvier (4) were able to isolate *B. paratyphosus* from the intestinal tract 24 hours after intravenous injection. Breton, Bruyant and Mézie (5), repeating the experiments of Hess, recovered *B. prodigiosus* from the bile from ½ hour to 24 hours after intravenous injection of 1 mg. of a 24 hour culture. They cite an experiment in which they recovered tubercle bacilli from the bile at the end of 6 hours.

Though the literature[1] on the elimination of tubercle bacilli by tuberculous patients and cattle is extensive, there is no study which

[1] The literature on the elimination of tubercle bacilli in man and cattle is reviewed by Calmette in "Tubercle bacillus infection and tuberculosis in man and animals," translation by Soper, W. B., and Smith, G. H., Baltimore, 1923, p. 444.

demonstrates that tubercle bacilli are eliminated in the feces of patients or cattle when tuberculous lesions are not in open communication with the surface. When a definite communication exists between a caseous focus and the intestinal tract, it is obvious that tubercle bacilli will be eliminated in the feces.

The Elimination of Tubercle Bacilli in the Feces of Experimentally Infected Guinea Pigs.

An attempt was made to determine the significance of fecal contamination as a source of infection in the spread of spontaneous tuberculosis in guinea pigs.

Experiment 1.—Five guinea pigs were injected intraperitoneally with 0.001 mg. of a glycerol agar transplant 3 weeks old made from a virulent human strain of tubercle bacilli (P 15-B). One normal guinea pig received 0.01 mg. of the same culture subcutaneously. These animals were used in the epidemiological experiments described in a previous paper (1). The feces of these six guinea pigs were tested every week.

If the abdomen of a guinea pig is gently massaged 1 hour after feeding, fecal boluses are readily passed. These boluses, in the absence of an enterocolitis, are firm and uniform in size. Two boluses from each of the guinea pigs tested were collected in sterile Petri dishes. The feces of each animal were ground up in a test-tube with a small quantity of sterile saline solution and the emulsion was filtered through several layers of gauze. Twice the volume of 3 per cent sodium hydroxide was added to the tube and the whole was incubated during 1 hour at 37°C. in a water bath. The emulsion was then centrifuged and the supernatant liquid decanted. The sediment was neutralized against phenolphthalein solution (1 per cent) with 6 per cent hydrochloric acid. The volume was then brought to 5 cc. with sterile saline solution. 2 cc. of this emulsion was injected into the subcutaneous tissue of the thigh of each of two guinea pigs. Monthly intradermal tuberculin tests with 0.02 cc. of "old tuberculin" were made on all the test pigs. All test pigs were killed at the end of 3 months. The results of this experiment are given in Table I. All of the animals of which the feces were tested died of generalized tuberculosis.

Tubercle bacilli have passed through the tissues of the normal animal and are eliminated during the 1st week of infection. The development of immunity against the microorganism may later cause fixation of the bacilli within the tissue thus preventing their elimination. With dissemination of the infection, bacilli are again eliminated.

In the following experiment all animals were inoculated with the

TABLE I.

Elimination of Tubercle Bacilli in the Feces of Experimentally Infected Guinea Pigs.

	Tubercle bacilli in the feces tested at intervals indicated below:						
	1 wk.	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.	
Guinea Pig 1, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	0	0	+	X	X	Died at end of 7 wks.
Guinea Pig 2, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	+	X	0	0	0	Died after 13 wks.
Guinea Pig 3, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	0	0	+	+	X	Died after 8 wks.
Guinea Pig 4, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	0	0	0	0	X	Died after 14 wks.
Guinea Pig 5, 0.001 mg. *B. tuberculosis*, intraperitoneally	+	0	0	X	X	X	Died after 4 wks.
Guinea Pig 6, 0.01 mg. *B. tuberculosis*, subcutaneously	X	X	0	+	+	X	Died after 14 wks.

X = no test made on this date. + = tuberculosis present in the test animals.
0 = no evidence of tuberculosis in the test animals.

TABLE II.

Elimination of Tubercle Bacilli in the Feces of Experimentally Infected Guinea Pigs

	Tubercle bacilli in the feces tested at intervals indicated below:							
	Before inoculation	1 day	2 days	4 days	1 wk.	3 wks.	5 wks.	
Guinea Pig 7, 0.001 mg. *B. tuberculosis*, intraperitoneally	0	0	0	0	0	0	0	Died after 51 days of tuberculosis
Guinea Pig 8, 0.001 mg. *B. tuberculosis*, intraperitoneally	0	0	0	0	0	0	0	Killed after 56 days of generalized tuberculosis
Guinea Pig 9, 0.001 mg. *B. tuberculosis*, intraperitoneally	0	+	0	0	0	0	+	Died after 56 days of tuberculosis
Guinea Pig 10, 0.001 mg. *B. tuberculosis*, intraperitoneally	0	0	0	0	0	0	+	Died after 52 days of tuberculosis

dose of tubercle bacilli used in Experiment 1 (Guinea Pig 6 excepted).

Experiment 2.—Four guinea pigs received 0.001 mg. of tubercle bacilli (P 15-B) injected into the peritoneal cavity. After intervals of 1, 2, 4, 7, 21 and 35 days the feces of each of these animals were collected and injected into two normal guinea pigs.

The foregoing experiments were not carried beyond the 6th week, and the following experiment was performed in order to collect more information concerning the elimination of tubercle bacilli.

Experiment 3.—Four guinea pigs with negative tuberculin reactions were inoculated with human tubercle bacilli of a glycerol agar growth (P 15-B) 3 weeks old, as follows:

Guinea Pig 11 received 0.001 mg. intraperitoneally and died during the 9th week.

Guinea Pig 12 received 0.00001 mg. intraperitoneally and died at the end of the 11th week.

Guinea Pig 13 received 0.01 mg. subcutaneously in the left thigh, and died at the end of the 7th week.

Guinea Pig 14 received 0.0001 mg. subcutaneously in the left thigh, and died at the end of the 13th week.

All of these animals died of disseminated tuberculosis. On the day when the feces were collected the animals were tested with 0.02 cc. of tuberculin. The procedure was that described in Experiment 1.

None of the guinea pigs inoculated with feces taken during the first 4 weeks of the infection developed tuberculosis. It is however noteworthy that the only animal in Experiment 3 comparable with those of Experiments 1 and 2 is Guinea Pig 11, but in this instance an early elimination of tubercle bacilli in the feces did not occur. Guinea Pig 12 received a very small intraperitoneal injection and in Guinea Pigs 13 and 14, injections were subcutaneous.

Tubercle bacilli were eliminated in the feces of the animals in Experiment 3 during the last few weeks of the infection. The elimination of tubercle bacilli occurred just before and during the interval when the tuberculin reaction was diminishing in intensity or was negative. The number of experiments, however, is not sufficient to establish definitely the relation of the tuberculin reaction to the terminal elimination of tubercle bacilli. The negative tuberculin reaction has been a manifestation of overwhelming infection and only after the disease is apparently well advanced and disseminated, are tubercle

bacilli eliminated in the feces in sufficient quantities to infect guinea pigs.

The Elimination of Tubercle Bacilli in the Bile of Experimentally Infected Guinea Pigs.

There have been numerous studies of the elimination of tubercle bacilli in the bile.

Calmette and Guérin (6) found that tubercle bacilli are eliminated in the bile of tuberculous rabbits. They injected a series of rabbits intravenously with 10 mg. of bovine tubercle bacilli and killed them at intervals of 24 and 48 hours, 3, 4, 5, 6 and 7 days. In each instance, the contents of the gall bladder were aspirated and injected into guinea pigs. Tubercle bacilli were recovered from the bile obtained on the 3rd and 7th days. By establishing a permanent biliary fistula in a heifer (7), they were able to make daily tests of the bile aspirated through the fistulous opening. Following intravenous injection of 3 mg. of virulent bovine tubercle bacilli, they found that tubercle bacilli were eliminated in the bile only after 19 days. The heifer died of miliary tuberculosis 26 days after inoculation. In a series of twenty-four guinea pigs with disseminated tuberculosis killed at a time when death was expected as a result of the infection, Remlinger (7) tested the contents of the gall bladder for tubercle bacilli by direct inoculation into guinea pigs and observed eighteen positive results. Sasano and Medlar (8) found that the appearance of tubercle bacilli in the bile of experimentally infected guinea pigs is indicative of tuberculosis of the liver and is usually evidence of an ulcerative tuberculous lesion of the biliary system. From the time the bile duct becomes involved until death, there is a continuous elimination of tubercle bacilli into the intestinal tract (9). The exact time of appearance of the tubercle bacilli in the bile is not stated in their work.

Tubercle bacilli have been found in the bile of patients dying of pulmonary tuberculosis by Fraenkel and Krause (10) and by Corper, Simmons and Freed (11). In patients suffering with tuberculosis tubercle bacilli have been discovered in the bile aspirated through a duodenal tube, by Carnot and Libert (12), Freed and Black (13), Olmer and Crémieux (14) and others. There is, however, no evidence recorded in the literature which conclusively demonstrates the elimination of tubercle bacilli through the biliary system in the absence of lesions of the liver or of the biliary tract.

To explain the spread of spontaneous tuberculosis it is desirable to determine when tubercle bacilli appear in the bile of infected guinea pigs and to compare the elimination in the bile with that in the feces. It may be that this is the main source of tubercle bacilli when the disease is disseminated and involves the liver. The attempt has been

TABLE III.

Elimination of Tubercle Bacilli in the Feces of Experimentally Infected Guinea Pigs.

	Tubercle bacilli in the feces tested at intervals indicated below:																	
	24 hrs. before inoculation	24 hrs. after inoculation	2 days	4 days	6 days	11 days	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.	7 wks.	8 wks.	9 wks.	10 wks.	11 wks.	12 wks.	13 wks.
Guinea Pig 11 0.001 mg. *B. tuberculosis*, intraperitoneally	0	0	0	0	0	0	0	0	0	0	+	0	+					
Tuberculin reaction	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1+	3+	2+	4+	4+	3+	1+					
Guinea Pig 12 0.00001 mg. *B. tuberculosis*, intraperitoneally	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+		
Tuberculin reaction	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1+	2+	4+	4+	3+	4+	3+	1+	Neg.		
Guinea Pig 13 0.01 mg. *B. tuberculosis*, subcutaneously	0	0	0	0	0	0	0	0	0	0	+	+						
Tuberculin reaction	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1+	2+	2+	3+	4+	2+						
Guinea Pig 14 0.0001 mg. *B. tuberculosis*, subcutaneously	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+
Tuberculin reaction	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1+	1+	3+	4+	4+	3+	3+	2+	Neg.	Neg.	Neg.

The intensity of the tuberculin reaction is indicated by 1+, 2+, 3+ or 4+.

made to study the elimination of tubercle bacilli in the bile of infected guinea pigs throughout the course of the infection.

Experiment 4.—Nine normal guinea pigs of the same weight and age and from the same stock were inoculated subcutaneously with 0.001 mg. of the strain of virulent human tubercle bacilli (P 15-B) used in the previous experiments. These animals were killed at intervals of 24 and 48 hours, 4, 7, 14, 21, 28 and 42 days. The last animal died after 44 days. The animals were chloroformed and the abdomen opened with aseptic conditions. The gall bladder was brought prominently into view, the surface thoroughly seared and the entire contents of the bladder aspirated. The organs of these animals were carefully examined and sections were made from the liver, kidney and site of inoculation.

TABLE IV.

	Interval between infection and death in days	Tubercle bacilli in bile	Tubercle bacilli in urine	Remarks
Guinea Pig 15 not infected		0	0	No tuberculosis
Guinea Pig 16	1	0	0	No tuberculosis
Guinea Pig 17	2	0	0	No tuberculosis
Guinea Pig 18	4	0	0	No tuberculosis
Guinea Pig 19	7	0	0	No tuberculosis
Guinea Pig 20	14	+	0	Tuberculous lesion at site of injection and in regional lymph nodes
Guinea Pig 21	21	+	0	Tuberculous lesion at site of injection and in inguinal, iliac and lumbar lymph nodes. Spleen enlarged but liver apparently normal
Guinea Pig 22	28	+	0	Same as Guinea Pig 17
Guinea Pig 23	42	+	0	Tuberculosis of spleen, liver and lungs
Guinea Pig 24	44	+	+	Tuberculosis of lymph nodes, liver, spleen and lungs

The total volume was brought up to 4 cc. with sterile saline solution and injected subcutaneously into two guinea pigs. These test guinea pigs were killed as soon as the tuberculin reaction was strongly positive or at the end of 3 months. The results of this experiment are given in Table IV.

Tubercle bacilli were eliminated in the bile of the experimentally infected guinea pigs from the 14th day until death.

The following experiment was made to determine whether tubercle

bacilli were eliminated with the bile during the first few hours following inoculation.

Experiment 5.—Guinea pigs were injected into the peritoneal cavity with 0.01 mg. of a culture of *B. tuberculosis*, and after intervals of 4, 8, 12, 24 and 48 hours the bile of each of these pigs was collected and injected into two normal pigs. The results of this experiment are given in Table V.

After subcutaneous inoculation of tubercle bacilli with the dose used (0.01 mg.) tubercle bacilli have appeared in the bile only once within the first 2 days after injection.

TABLE V.

	Interval between infection and death in hrs.	Tubercle bacilli in bile
Guinea Pig 25	4	0
Guinea Pig 26	4	0
Guinea Pig 27	8	0
Guinea Pig 28	8	0
Guinea Pig 29	12	0
Guinea Pig 30	12	0
Guinea Pig 31	24	+
Guinea Pig 32	24	0
Guinea Pig 33	48	0
Guinea Pig 34	48	0

Elimination of Tubercle Bacilli in the Urine of Experimentally Infected Guinea Pigs.

Remlinger (7) in a series of twenty-four guinea pigs with disseminated tuberculosis killed at a time when death was expected found that the urine aspirated from the urinary bladder in half of the animals, contained virulent tubercle bacilli, demonstrated by animal inoculation.

Although there are numerous clinical studies demonstrating the presence of tubercle bacilli in the urine of individuals in whom there has been no evidence of renal tuberculosis, it is not improbable that transient bacteremia has caused minute focal lesions of renal glomeruli which have healed or have been overlooked at autopsy.

An attempt has been made to determine whether tubercle bacilli eliminated with the urine of tuberculous guinea pigs experimentally

infected for the elimination of tubercle bacilli in the urine, may facilitate the spread of spontaneous tuberculosis. In guinea pigs used in Experiment 4, the urinary bladder was seared and the urine was aspirated aseptically. The total volume from each animal was brought up to 4 cc. with sterile saline solution and injected subcutaneously into two guinea pigs. The results of this experiment are given in Table IV.

The urine aspirated from the bladder of infected guinea pigs did not contain tubercle bacilli until the terminal stage of the infection. Guinea Pig 24 died 44 days after inoculation and the urine aspirated from its bladder caused tuberculosis in guinea pigs.

DISCUSSION.

In the course of experimental infections in animals and in spontaneous disease in man, tubercle bacilli doubtless appear intermittently in the blood stream.

Calmette (15) in a review of the literature of bacteremia with tuberculosis maintains that with experimental tuberculous infection of animals bacteremia it present before a lesion appears at the site of inoculation and continues throughous the infection. A large quantity of blood may be necessary to show presence of tubercle bacilli. Calmette demonstrated tubercle bacilli in the blood stream by direct transfusion of 10 cc. of blood from a tuberculous into a normal guinea pig. This huge quantity of blood is approximately equivalent to a liter of human blood.

Bergeron (16) states that bacteremia in experimental tuberculosis of rabbits occurs soon after injection and is ephemeral.[2] Haga (18) tested the blood of guinea pigs infected subcutaneously with virulent human tubercle bacilli. He found by animal inoculation and by staining methods that tubercle bacilli were present in the blood stream during the first 10 days after inoculation in five of twelve experiments. The number of positive results diminished from the 10th to the 30th day, and almost wholly disappeared during the interval from the 30th to the 40th day. From the 40th day until death bacilli were again discovered in the blood stream in increasing numbers and were present in two-thirds of the animals examined. In 66 per cent of instances, they appeared between the 50th and the 70th day after inoculation.

In normal rabbits, Opie (19) has demonstrated that antigens such as horse serum or egg white are demonstrable in the blood during a period from approximately 7 to 9 days after injection. "With the progress of immunization injected antigen

[2] The literature of the subject is thoroughly reviewed by Löwenstein (17) and by Calmette (15).

exhibits a decreasing tendency to find its way into the circulating blood so that in a well immunized animal horse serum or egg white injected into the subcutaneous tissue is not demonstrable in the blood serum even if tests are made at intervals from 1 to 24 hours after injection." In a subsequent study it has been found that the antigen introduced into an immune animal is fixed at the site of injection (20).

In the studies of Krause (21) and Willis (22) on the spread of tubercle bacilli in normal and immune guinea pigs infected by subcutaneous inoculation, it has been demonstrated that tubercle bacilli inoculated intracutaneously or subcutaneously into normal guinea pigs are carried to the regional lymph node within an hour, and within 3 or 4 days they have been disseminated throughout the body and may be isolated from the lungs, hilum lymph nodes and other tissues. In immune animals their spread is greatly retarded. They remain fixed at or near the site of entry during about 7 days, do not reach the regional lymph nodes until 2 weeks after infection and are not disseminated throughout the body until 2 or 3 weeks after infection. "The immune state (that is, the allergic reaction) operates to retard the spread of the bacilli . . . . rather than to inhibit . . . . their activities or their multiplication" (21).

The observations described in this paper show that tubercle bacilli may be eliminated with the feces during the 1st week following inoculation. At a later period elimination ceases and does not begin again until the disease is far advanced. Elimination by way of the bile occasionally occurs immediately after inoculation, but is insignificant until tuberculosis is well established in the body. Elimination by way of the urine has occurred only when the disease has reached its terminal stage.

Tubercle bacilli appear in the blood stream of infected guinea pigs during the first 10 days following subcutaneous injection, but gradually disappear and reappear after about 40 days (Haga). Elimination of microorganisms in the bile of rabbits injected intravenously with large quantities of tubercle bacilli occurs within 3 days (Calmette and Guérin), and as early as 6 hours (Breton, Bruyant and Mézie). The evidence presented by these investigators and the observations reported in this paper show that tubercle bacilli introduced forcibly into the tissues of normal animals enter the blood stream and are eliminated in the feces before immunity against the microorganism is established. With the progress of immunization the bacilli become fixed in the tissues, are no longer capable of permeating intact mucous membranes and do not appear in the feces. When the infection is widely disseminated, resistance is overcome and the elimination of tubercle bacilli recurs.

CONCLUSIONS.

1. After intraperitoneal inoculation of guinea pigs with tubercle bacilli, the microorganisms may appear in the feces and elimination may persist during 1 week. Subsequently during several weeks no tubercle bacilli can be recovered from the feces.

2. When tuberculosis is widely disseminated in the body of guinea pigs, tubercle bacilli are discharged with the feces.

3. Tubercle bacilli are occasionally eliminated in the bile of guinea pigs immediately after inoculation but are almost constantly found in the bile after several weeks when the disease is well established.

4. Tubercle bacilli are eliminated in the urine of experimentally infected guinea pigs only when infection is far advanced.

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BACTERIOPHAGE ISOLATED FROM THE COMMON HOUSE FLY (MUSCA DOMESTICA).

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INTRODUCTION.

It is known, through the work of others, that the house fly, when fed cultures of various pathogenic bacteria, harbors them for a longer or shorter time but that they finally disappear entirely. Various explanations of this elimination have been offered or are suggested by the facts presented. Wollman (1) regarded the process as entirely mechanical. In the case presented by Jones and Little (2) an actual rapid sterilization was observed in which more subtle factors are obviously concerned.

Glaser and Sanderson, in unpublished observations from this laboratory, found in house flies bacteriophage active against *Staphylococcus muscæ*, a microorganism which gives rise to a definite disease of this insect (Glaser (3)). This observation offered the suggestion that bacteriophage might be found more widely active and might be concerned in the natural process of removal of foreign bacteria when these are fed to the fly. Duncan (4) has recently studied the same problem and discovered a bactericidal principle active against many species of bacteria in the gastrointestinal tracts of various insects, the fly among them. The references above generally commented on are more specifically as follows.

Graham-Smith (5) found that, with flies fed on cultures, *B. prodigiosus* (page 96) could survive in the crop only about 17 days. *B. enteritidis* Gärtner (page 146) could be recovered from the gut contents up to the 7th day. *B. typhosus* (page 130) up to the 6th day, and *V. choleræ* (page 173) for 2 days after ingestion by the insect. Manson-Bahr (6) found that *B. dysenteriæ* Shiga, from a culture, survived in the gut contents of flies 4 days. Naturally infected flies, he thought, carried the organ-

isms for a much longer time. Wollman (1) observed that flies fed on cultures of *B. typhosus* and *B. dysenteriæ* and then transferred daily to aseptic surroundings became free of the specific bacteria in from 8 to 10 days. Jones and Little (2), in conducting an investigation on infectious ophthalmia of cattle, made the observation that the causative diplobacillus was not capable of surviving as long as 5 minutes in the gastrointestinal tract of the house fly. Duncan (4) isolated a bactericidal principle from the alimentary tracts of a number of insects and arachnids. He included *Musca domestica* in the series of insects found by him to contain this bactericidal principle in their gastrointestinal contents. He considered that this principle might be a bacteriophage but was forced to discard the possibility as the substance he described exhibited none of the essential properties of this principle.

The experimental work recorded in the following pages was conducted with the purpose of determining any bactericidal action which might pertain to the house fly, and its result has been to identify bacteriophage very active against a number of bacteria, as well as an inhibitory substance, not identified with bacteriophage, and active still more widely.

EXPERIMENTAL.

1000 house flies were obtained from the vicinity of a hog lot, etherized and ground in a mortar with physiological salt solution in the proportion of 10 flies per cc. This mixture was filtered first through paper, then through a Berkefeld N filter. A clear, dark, straw-colored fluid was obtained which gave no bacterial growth when incubated on the ordinary culture media. This fluid was kept at refrigerator temperature and was used as the starting point for most of the experimental work here reported.

Two organisms were used at the outset to determine whether or not the fly filtrate just described contained substances which were either bactericidal or capable of inhibiting bacterial growth. One of these was a non-mucoid strain of *B. coli* 223 of calf origin (7), and the other *B. paratyphi* Type I of guinea pig origin (8).

Plain bouillon (pH 7.5), 5 cc. per tube, was used as the culture medium and the fly filtrate was added to the bouillon before inoculation. 1, 0.5, 0.25, and 0.1 cc. amounts of fly filtrate were added to four separate tubes in each series and one tube in each was kept as a control. One loopful of a 24 hour bouillon culture was used in making each inoculation. The tubes were read at 6 and 24 hours with the results given in Table I.

This experiment made it evident that the fly filtrate contained some substance which was inhibitory or bactericidal for *B. coli* (calf) but

which exerted no such action upon *B. paratyphi* Type I (guinea pig). This apparent specificity suggested the possibility that the factor might be bacteriophage. Each tube of the two sets of cultures was therefore passed through a Berkefeld N filter and the experiments were

TABLE I.

	B. paratyphi Type I (guinea pig)		*B. coli* (calf)	
	6 hrs.	24 hrs.	6 hrs.	24 hrs.
cc.				
1	Moderately turbid	Turbid	Clear	Clear
0.5	" "	"	"	"
0.25	" "	"	"	"
0.1	" "	"	"	"
Control	" "	"	Moderately turbid	Turbid

TABLE II.

Dilution	*B. paratyphi* Type I (guinea pig)			*B. coli* (calf)		
	6 hrs.	24 hrs.	48 hrs.	6 hrs.	24 hrs.	48 hrs.
10^{-1}	C.*	M.T.	M.T.	C.	V.S.T.	S.T.; agglutinated
10^{-2}	"	"	"	"	"	" "
10^{-3}	"	"	"	"	"	" "
10^{-4}	"	"	"	"	S.T.; agglutinated	M.T.; "
10^{-5}	S.T.	S.T.	S.T.	"	" "	" "
10^{-6}	"	"	"	"	" "	" "
10^{-7}	M.T.	"	"	"	" "	" "
10^{-8}	"	"	"	S.T.	" "	" "
10^{-9}	"	"	"	"	" "	" "
Control	"	T.	T.	M.T.	T.	T.

* C. = clear; V.S.T., very slightly turbid; S.T., slightly turbid; M.T., moderately turbid; T., turbid.

repeated using the bouillon filtrate. The dilutions were made this time from tube to tube increasing by successive powers of ten. A single pipette was used for each series. The results are given in Table II.

After 48 hours incubation, each tube of these two sets of cultures was passed through a Berkefeld N filter and the experiment repeated with results similar to the above.

TABLE III.

Dilution	*B. typhosus* (Rawlings)			*B. paratyphi* Type I (guinea pig)			*B. coli* (calf)			*Staphylococcus muscæ*		
	6 hrs.	24 hrs.	48 hrs.	6 hrs.	24 hrs.	48 hrs.	6 hrs.	24 hrs.	48 hrs.	6 hrs.	24 hrs.	48 hrs.
10^{-1}	C.*	C.; Sed.	C.; Sed.	C.	M.T.	M.T.	C.	C.	S.T.	C.	C.	C.
10^{-2}	"	" "	" "	"	"	"	"	"	"	"	"	"
10^{-3}	"	" "	" "	S.T.	"	S.T.	"	S.T.	"	"	"	"
10^{-4}	"	" "	" "	"	"	"	"	"	"	"	"	"
10^{-5}	"	" "	" "	T.	"	M.T.	"	"	"	"	"	"
10^{-6}	"	" "	" "	"	T.	"	"	"	"	"	"	"
10^{-7}	"	" "	" "	"	"	"	S.T.	"	"	"	"	"
10^{-8}	"	S.T.; "	S.T.; "	"	"	"	"	"	"	S.T.	"	"
10^{-9}	S.T.	T.	T.	"	"	T.	M.T.	M.T.	"	"	"	"
10^{-10}	"	"	"	"	"	"	"	T.	T.	"	M.T.	M.T.
10^{-11}	M.T.	"	"	"	"	"	"	"	"	"	T.	T.
10^{-12}	"	"	"	"	"	"	"	"	"	"	"	"
Control	"	"	"	"	"	"	"	"	"	"	"	"

* C. = clear; S.T., slightly turbid; M.T., moderately turbid; T., turbid; Sed., sedimentary growth.

Plates were next made and these showed typical plaque formation in both series. Individual colonies on solid media showed notched and stellate forms. The inhibition of growth in series by dilutions of the filtrates and the formation of plaques definitely characterizes the action as that of bacteriophage.

The activity of the original fly filtrate against a wider range of bacteria was then tested. Preliminary determinations were carried out using a single pipette for each set of dilutions, while the experiments to determine the definite end-points of activity, those given in Tables III and IV, were done using a fresh pipette for each dilution.

TABLE IV.

Organism	Highest dilution showing inhibition of growth at 6 hrs.
Staphylococcus aureus	1:1000 (complete)
Streptococcus C 54	1:100 "
" 744	1:10 (complete). 1:10,000 (partial)
" C 55	1:10,000 (partial)
Pneumococcus I	1:10 (complete). 1:10,000 (partial)
" II	1:10,000 (complete)
Bacillus of swine plague	1:1000 (partial)
Vibrio choleræ	No inhibition of growth
Friedländer's bacillus	" " " "
Bacillus proteus	" " " "

Whenever evidence of inhibition of growth was obtained filtrates were made and tested in order to develop any potential transmission in series. Inhibition, displayed in successive filtrates, was thus uncovered in the case of *B. typhosus* (Rawlings) and *Staphylococcus muscæ* in addition to the two species previously considered. The complete record, as finally determined for the four species, is shown in Table III.

The original filtrate inhibited the growth of certain other species in various dilutions but filtrates in these cases had no inhibitory action. The end-point of activity for these bacteria and the species for which no inhibitory action could be demonstrated are contained in Table IV.

The bacteriophage isolated was very active against *Staphylococcus muscæ*, giving complete inhibition of growth to a dilution of 10^{-9}. The lysis of this organism was complete and permanent and no secondary growth ever occurred in the tubes that were completely lysed at 48

hours. It was fairly active against *B. typhosus* (Rawlings), giving lysis to a dilution of 10^{-8}, but in the case of this organism all lysed tubes showed some secondary growth in the form of a sediment. Against *B. coli* (calf) it was also fairly active, giving evidence of lysis in a dilution of 10^{-9}. With this organism a secondary growth occurred in 24 hours also. The lytic principle active against *B. paratyphi* Type I (guinea pig) was a relatively weak one and a secondary growth always occurred. Lysis in this case seemed to progress for at least 48 hours but complete clearing of the cultures never resulted.

The nature of the inhibitory action which differs so definitely from bacteriophage is of considerable interest. One possibility seemed to be that it might be related as a precursor or "building stone" for the bacteriophage. To test this, one of the species susceptible to inhibition, but for which bacteriophage was not developed, was fed to flies for a period. These flies were extracted as before and the filtered extract was tested for the characteristic transmissible lysis for the bacterium fed. The result was negative. The detailed experiment follows.

Streptococcus C 55, a non-hemolytic strain originating in bovine mastitis (9), which was inhibited but not susceptible to lysis by the bacteriophage in the original fly filtrate, was used. About 200 flies were placed in a large glass jar and fed bouillon cultures of Streptococcus C 55 daily for 8 days. At the end of this time the flies were etherized and ground in a mortar with physiological salt solution using 5 flies per cc. This material was passed through a Berkefeld N filter for sterilization and, from the filtrate, attempts were made to obtain bacteriophage capable of lysing cultures of Streptococcus C 55. These attempts were all unsuccessful and no bacteriophage active against this organism could be obtained.

DISCUSSION.

Physiological salt solution extracts of the house fly present bacteriolytic or inhibitory phenomena of two types which may have a bearing on the inability of certain pathogenic microorganisms to exist for more than a short period of time in the gastrointestinal tract of the insect. Bacteriophage active against at least four species of bacteria was found in a salt solution extract of flies; and another substance, growth-inhibiting, but not showing the essential characteristics of bacteriophage, was also present. This was active against four additional species.

In so far as relates to the bacteriophage, it is very likely that the fly filtrate contains a mixture of lytic principles, rather than a single bacteriophage capable of causing lysis of the four species. Thus neither the broth filtrate active against *B. coli* (calf) nor the one active against *B. paratyphi* Type I (guinea pig) had any lytic action on *Staphylococcus muscæ*. The filtrate active against *B. paratyphi* Type I (guinea pig) was also strongly lytic for *B. typhosus* (Rawlings). The filtrate active against *B. coli* (calf) was somewhat active for *B. typhosus* (Rawlings), giving lysis to a dilution of 10^{-4}. The filtrate active against *Staphylococcus muscæ* failed completely to cause lysis of *B. typhosus* (Rawlings). The filtrate active against *B. coli* (calf) caused no lysis of *B. paratyphi* Type I (guinea pig). No effort was made to adapt a lytic principle, active against one organism, to another of the group.

Because of the possibility that the non-bacteriophagic growth-inhibiting substance might be a precursor to true bacteriophage, the feeding experiment described was planned. No bacteriophage against the streptococcus fed could be obtained in this way. It is very likely that this non-bacteriophagic growth-inhibiting factor is the same as that observed by Duncan (4).

Within the range of the experimental observations, the lytic principle present in the fly filtrate may have been obtained either from the external parts of the fly or from its digestive tract. A much more elaborate technical procedure would be required to make this discrimination.

SUMMARY AND CONCLUSIONS.

1. Bacteriophage active against four species of bacteria was found in a salt solution extract of house flies.

2. A growth-inhibiting principle, not bacteriophage, active against four other species of bacteria was found to be present in the same extract.

3. An attempt to secure streptococcus bacteriophage by feeding to flies a streptococcus susceptible to the inhibitor but not to the bacteriophage of this filtrate was unsuccessful, indicating that the two activities are quite unrelated.

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ON THE INFLUENCE OF ACID GROUPS ON THE SEROLOGICAL SPECIFICITY OF AZOPROTEINS.*

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PLATE 31.

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In previous work (2–5) investigations were made of the relation between chemical constitution and serological specificity by compounding various substances of simple chemical structure with proteins and using the resulting combinations as antigens. The coupling of proteins with diazonium derivatives of aromatic substances offered itself as the most suitable means. The immune sera obtained were tested by the precipitin reaction. In order to avoid species-specific reactions on the protein component the test antigens generally were prepared with a protein from another species than that employed for the immunizing antigens. The principal result of these studies was the observation that the immune sera exhibit a specificity which is determined by the chemical structure of the simple compounds attached to the protein.[1]

Aside from the phases already dwelt upon there are others to which the method outlined is applicable. The present paper deals with the influence of acid groups on specificity. Reference has been made to this question in one of the papers quoted (3).[2] For our purpose antigens were prepared with chemicals with and without acid groups; after immunization with these substances the immune sera were tested with both types of antigens.[3]

* Twenty-second paper on antigens and specificity. *Cf.* Reference 1.

[1] For a fuller review we refer to "The chemical aspects of immunity," by H. Gideon Wells, American Chemical Society Monograph Series, New York, 1925, page 77.

[2] Page 387.

[3] For the sake of brevity these will be referred to in the following as acid and non-acid antigens and immune sera respectively.

EXPERIMENTAL.

Preparation of the Antigens for Immunization.—As an example the method used for aniline is given. The other substances were taken in quantities equivalent to that of the aniline.

1.2 gm. of aniline were dissolved in 10 cc. of water and 5 cc. of 7 N HCl and diazotized with the necessary amount of sodium nitrite at a temperature of 0–5°C., with starch iodide paper as indicator. The diazo solution was diluted with ice water to a volume of 100 cc. and poured into a mixture of 100 cc. of horse serum and 100 cc. of normal sodium carbonate. The solution must give a strongly alkaline reaction with phenolphthalein. Coupling was allowed to take place for 10 minutes at 0–5°C. By acidification with hydrochloric acid the azoprotein was precipitated and after filtration it was suspended in a small amount of water. On addition of some normal sodium hydroxide and vigorous stirring it became viscous or jelly-like. A large quantity of alcohol was added and subsequently enough hydrochloric acid to flocculate the material.

The precipitate was filtered and treated twice again in the same manner. In order to remove the alcohol it was brought into solution with alkali as before and after dilution it was reprecipitated with hydrochloric acid and filtered. After grinding in a mortar the substance was dissolved in water at alkaline reaction. Finally the reaction was adjusted with hydrochloric acid to faint alkalinity or neutrality.

The volume was made up to 190 cc. with distilled water and the necessary amount of a salt solution to make the ultimate salt concentration approximately 1 per cent. 10 cc. of a 5 per cent phenol solution were added. The presence of some suspended azoprotein in the solution does not affect its use for the immunization.

The other preparations were made in a similar way. Some of them such as the azoproteins from ortho- and para-chloroaniline are still less soluble, therefore a great part of the substance remains in suspension. The diazotization of para-nitroaniline was carried out by adding at room temperature the sodium nitrite solution to a suspension of the finely powdered substance containing the required amount of hydrochloric acid. Small amounts of undissolved material were removed by filtration. This same method was applied to ortho-nitroaniline and 1, 2, 5-nitrotoluidine which were only used for the preparation of test antigens. In the case of the amino acids[4] as para-arsanilic acid, para-aminobenzoic acid, etc., the azoproteins can be dissolved without difficulty. For coupling diazotized ortho-aminobenzoic acid with horse serum, 50 cc. of normal NaOH per 100 cc. serum were used instead of sodium carbonate.

Immunization.—Eight rabbits were injected intraperitoneally with 15 cc. or less of each antigen at weekly intervals. At least two immune sera of sufficient strength were obtained after three to six injections in every case. Only one serum was obtained in the instance of para-chloroaniline.

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[4] For further details see Reference 3, pages 354–364.

Antigens for the Tests.—The azoproteins for the test solutions were prepared with chicken serum in place of horse serum in the manner described above. The diazo solution (in a few instances containing undissolved diazo compound) was added to a mixture of serum and sodium hydroxide (1 part of normal sodium hydroxide to 2 parts of serum) and the coupling allowed to take place at room temperature for half an hour. After precipitation with dilute hydrochloric acid the azoprotein was filtered and washed with water.

There was some difficulty in that the preparations did not yield clear solutions or were only sparingly soluble. This was overcome by treatment of the azoprotein with alkali. An amount of the antigen corresponding to 5 cc. serum was ground in a mortar, made up to 10 cc. with water and 10 cc. of normal NaOH was added. After half an hour the mixture was neutralized with 10 cc. normal HCl, the precipitated azoprotein centrifuged and taken up in about 25 cc. saline containing 0.5 cc. normal NaOH. After a few minutes hydrochloric acid was added until the solution was only weakly alkaline to litmus paper and the volume made up to 30 cc. The solution to which 0.5 per cent phenol had been added was clarified by intense centrifugalization and filtration through kieselguhr paper.[5]

To make the tests comparable also the antigens with acid azo components were treated with alkali in the same manner (concerning their preparation see Reference 3, pages 362 and 363). To obtain solutions of antigens made from the halogen substituted anilines it was necessary to prolong the treatment with alkali. A quantity of the azoprotein corresponding to 5 cc. serum was ground and 20 cc. of normal NaOH added. The mixture was shaken with beads for 2 hours, neutralized and the precipitated azoprotein was taken up in 25 cc. of saline containing 0.5 cc. normal NaOH. After addition of hydrochloric acid until the solution was weakly alkaline to litmus it was brought up to 30 cc., centrifuged and filtered through kieselguhr paper.

The quantity of antigen present in the solutions was determined by precipitating the azoprotein with alcohol, drying and weighing.

The solutions were found to contain from about 20 to 45 mg. of dry material in 5 cc.

Tests.—The antigens were diluted to 1:100 of a 1 per cent solution and 0.2 cc. of this solution was used for the tests. The tests were kept at room temperature and were in some instances also read after standing overnight in the ice box. The intensity of the reactions is indicated as follows: 0, f.tr. (faint trace), tr. (trace), ±, +, +±, etc.

Tests with Azoproteins Made from Aniline and Various Substituted Anilines.

Tables I and II illustrate the action of various immune sera on the azoproteins prepared from aniline and a number of substituted anilines.

[5] Macherey, Nagel and Co., Düren, Germany.

TABLE I.

Of the immune sera 779 and 788 four drops were used, and of the others 2 drops. The readings were made after 1 hour at room temperature.[6]

Azoproteins prepared from chicken serum and:	Immune sera obtained from antigens made from horse serum and:									
	Aniline	Aniline	Ortho-chloroaniline	Ortho-chloroaniline	Para-toluidine	Para-toluidine	Para-toluidine	Para-nitroaniline	Para-nitroaniline	Para-chloroaniline
	No. 760	No. 761	No. 788	No. 793	No. 763	No. 766	No. 770	No. 775	No. 795	No. 779
Aniline	++±	+±	+±	++	+±	+±	++	+	±	+
o-Toluidine	++	±	++	++±	+±	+	+	±	f.tr.	±
o-Anisidine	+	0	+±	++	±	tr.	0	0	0	0
o-Nitroaniline	+	0	+	+	tr.	tr.	0	±	tr.	0
o-Chloroaniline	+±	±	++	++±	+	+	+	±	+	tr.
m-Toluidine	++	+	+±	++	+±	+	+±	+	+	+
m-Nitroaniline	+	tr.	+	+	+	±	+	+±	+±	±
m-Chloroaniline	++	+	+	+±	+±	+	+±	+	+	+
m-Bromoaniline	++	+	+	++	+±	+	+	+	+	+
p-Toluidine	+±	+	+	+	++	+±	++±	+	+	++
p-Anisidine	++	±	±	+	++±	+±	++±	+	+±	+±
p-Nitroaniline	+	±	±	tr.	+±	+	+±	++	++	++
p-Chloroaniline	++±	+	±	+	++	+±	++±	+±	++	++
p-Bromoaniline	++	+	+	+±	++	++	++±	+±	++	++
p-Iodoaniline	+±	+	+	+	++	+±	++±	+±	+±	++

TABLE II.

1,2,4-Nitrotoluidine	++	±	+	++	+±	+±	++	+±	+±	+
1,4,2-Nitrotoluidine	++	±	+±	+±	++	+	+±	+±	+±	+
1,2,5-Nitrotoluidine	+	tr.	±	tr.	+	tr.	±	+±	+±	±
1,3,4-Xylidine	+±	±	+±	++	++	+±	++	+	+	+±
1,4,5-Xylidine	+±	tr.	+±	++	+	+	+	+	+	tr.
1,4,2-Xylidine	+±	tr.	+±	++	+	+	+	+	±	tr.
Acetyl-para-phenylenediamine	0	0	0	0	0	0	0	0	0	0
Para-aminoacetophenone	tr.	0	0	0	+	tr.	±	+	±	0
Monomethyl-para-phenylenediamine	+±	+				+±	++	+±		

[6] The sera and the antigens are indicated by the names of the substances used in their preparation.

The experiments summarized in Table I show that the immune sera precipitate nearly all antigens. Also in most cases of negative reactions distinct precipitation occurred after standing overnight in the ice box and the weak reactions increased considerably in strength. Nevertheless a certain degree of specificity is to be observed.

The homologous reaction is always one of the strongest. The nature of the substituent is generally of but little influence; in the tests with

TABLE III.

4 drops were used of immune serum 729 and 2 drops of the others. The readings were made after 1½ hours at room temperature.

Immune sera obtained with antigens made from horse serum and:	Numbers of the immune sera	Azoproteins made from chicken serum and:										
		Para-aminobenzoic acid	Meta-aminobenzoic acid	Ortho-aminobenzoic acid	Para-arsanilic acid	Sulfanilic acid	Ortho-cinnamic acid	Aniline	Para-nitroaniline	Ortho-nitroaniline	Para-toluidine	Meta-toluidine
Para-amino-benzoic acid..	816	+++±	±	0	0	0	0	0	0	0	0	0
Para-amino-benzoic acid..	818	+±	0	0	0	0	0	0	0	0	0	f.tr.
Ortho-amino-benzoic acid..	729	0	0	++	0	0	0	±	±	±		±
Para-arsanilic acid.....	722	0	0	0	++++	0	0	0	0	0		f.tr.
Aniline	760	0	0	0	0	0	0	++±	+	+	+±	++
Para-nitro-aniline.....	775	0	0	0	0	0	0	+	+±	+±	+	+
Para-toluidine..	770	0	0	0	0	0	0	++	+±	0	++±	+±

several immune sera however the intensity of the reactions was more diminished by the presence in the azo component of a nitro or a methoxy group than by the other substituents tested.

More pronounced is the effect produced by the position of the substituents regardless of their nature. In the tests with immune sera against para substituted azoproteins the strength of the reactions decreases in a general way in the order para, meta, ortho. This sequence is reversed in the reactions with sera against the ortho sub-

stituted aniline. Observations similar to these have been made previously (3, 5). The aniline immune sera seem to react somewhat more weakly with ortho substituted antigens than with the others.

No definite regularities appear from the tests of Table II except for the negative or weak reactions with the antigens prepared from acetyl-para-phenylenediamine and para-aminoacetophenone. The other antigens behave similarly to those included in Table I.

Tests with Azoproteins Prepared from Amino Acids.

A considerable number of immune sera prepared with acid azoproteins were described previously (3).

TABLE IV.

2 drops of aniline immune serum No. 760 were used. Readings after 1 hour.

Azoproteins made from chicken serum and the following substances:	Antigen dilutions 1 to			
	100	300	1000	3000
Aniline	+++	++±	+	±
Ortho-nitroaniline	+	+	tr.	0
Meta-nitroaniline	++	+±	±	tr.
Para-nitroaniline	+±	+±	±	tr.
Ortho-anisidine	+	+	±	tr.
Para-anisidine	+±	+±	±	f.tr.
Para-aminobenzoic acid	tr.	0	0	0
Sulfanilic acid	tr.	0	0	0

The present experiments as recorded in Table III demonstrate the considerable influence of acid groups on the specificity.

Table IV gives the results of tests made with various concentrations of the antigens.

The sera prepared with "non-acid" antigens give no precipitation with the "acid" azoproteins.[7] In the converse experiment showing the action of the "acid" immune sera on the "non-acid" antigens, there were weak positive reactions especially with one of the four sera and

[7] Distinct precipitation occurs in higher concentration of the acid antigens. These reactions were shown to be non-specific flocculations since such reactions took place also with entirely unrelated immune sera as precipitins for rat, pig serum, etc.

some more weak reactions came up after keeping the tests overnight. The reactions with serum No. 729 increased somewhat in strength.

A representative experiment is illustrated by Figs. 1 and 2.

In conformity with the findings already referred to (3) the sera for acid antigens exhibit in general a considerably higher specificity than the others. This is further substantiated by a series of tests in which the antigens for the reaction *in vitro* were made with the same protein as that used for the immunization (horse serum). Also in this way the difference between the two sorts of antigens is brought out clearly (Table V; *cf.* Fig. 3) although the method would tend to destroy the specificity of the reactions, due to the fact that the immune sera react to a certain degree upon the protein part of the antigens.

TABLE V.

To 0.2 cc. of the diluted antigen 1 drop of immune serum was added. The first reading was taken after 5, the second after 15 minutes.

Immune sera obtained with antigens made from horse serum and:	Numbers of the immune sera	Antigens made from horse serum and:										Horse serum	
		Aniline		Para-toluidine		Para-nitroaniline		Para-arsanilic acid		Para-amino-benzoic acid			
Aniline.....	760	++±	+++	+±	++	++	+++	tr.	±	0	tr.	±	+
Para-toluidine..	763	++	++	++	++±	++	++	tr.	tr.	0	0	+	+
Para-nitro-aniline....	735	+±	++±	+	++	++	+++	tr.	±	0	±	tr.	±

Reactions with an Azoprotein Made from the Methyl Ester of Para-Aminobenzoic Acid.

In order to prove in a different way the results obtained the following experiments were carried out. Starting from the ester of an aromatic amino acid an azoprotein was prepared. Presumably this should react with the non-acid immune sera like the other non-acid antigens. If this azoprotein is treated in such a way as to bring about hydrolysis of the ester it should no longer react with such sera but should have acquired by virtue of the free carboxyl group the property of reacting with an immune serum specific for the corresponding acid.

The methyl ester of para-aminobenzoic acid was prepared according to the directions given by Einhorn (6). The recrystallized substance melted at 112°C.

Diazotization.—0.190 gm. of the finely powdered substance was suspended in 10 cc. of water. Normal hydrochloric acid and a 2 per cent solution of sodium nitrite were added alternately in small quantities keeping the solution distinctly acid to Congo red and adding the nitrite after a test with potassium starch iodide paper showed the disappearance of nitrous acid. The theoretical quantity of nitrite was used up. The solution was kept at 0–5°C. This diazo solution was added to a mixture of 10 cc. of chicken serum and about 7 cc. of normal sodium carbonate, cooled to 0–5°C. The mixture was distinctly alkaline to phenolphthalein but an excess of alkali was avoided to prevent hydrolysis of the ester. After coupling for 10 minutes at 0–5°C., and acidifying with hydrochloric the azoprotein was filtered and washed with water. It was made up with saline into a fine suspension (volume 20 cc.) and made slighly alkaline to litmus by addition of 0.1 cc. of normal sodium hydroxide. Undissolved material was removed by centrifuging. The azoprotein was precipitated by neutralization with hydrochloric acid and after centrifugalization it was redissolved in 10 cc. of saline with alkali, carefully avoiding an excess. The solution which was very faintly alkaline to litmus was centrifuged and filtered through kieselguhr paper. Its content of azoprotein was estimated in the manner described before. The test solution was made up to 1/100th of a 1 per cent solution.

The experiment turned out as was anticipated (Table VI). The solution of the ester-azoprotein was precipitated by aniline and para-toluidine immune sera and not by an immune serum for para-aminobenzoic acid except for a weak reaction observed when the tests were kept overnight in the ice box. This is in agreement with the behavior of other non-acid azoproteins.

Hydrolysis of the Ester-Azoprotein.—To 2 cc. of a 1 per cent solution of ester-azoprotein were added 2 cc. of 1/10 normal sodium hydroxide and samples of 0.5 cc. were taken from the mixture at various times, *i.e.*, immediately after mixing, and after being kept 5 minutes, 1, 4, 6, 8 and 20 hours, at room temperature. These samples were adjusted to faint alkalinity with 1/10 normal hydrochloric acid, the azoprotein thus being kept in solution.

Table VII gives the reactions with the two immune sera at various stages of the hydrolysis. The antigen concentration corresponded in all cases to 1/100 of a 1 per cent solution.

TABLE VI.

2 drops of immune serum were used. Readings after 1 hour and after standing overnight in the ice box.

Azoproteins made from chicken serum and:	Immune sera obtained from antigens made from horse serum and:							
	Aniline		Para-toluidine		Para-nitroaniline		Para-aminobenzoic acid	
	No. 760		No. 770		No. 775		No. 816	
	1 hr.	Night	1 hr.	Night	1 hr.	Night	1 hr.	Night
Aniline	++	++±	++	++±	+	++	0	tr.
Para-toluidine	+±	++	++	++±	+	++	0	tr.
Para-nitroaniline	+±	++	+±	++	+±	+++	0	±
Para-aminobenzoic acid	0	0	0	0	0	0	++	++++
Para-aminobenzoic ester	+	++	+	++	±	++	0	±

TABLE VII.

2 drops of immune serum were used. Readings after 1 hour at room temperature and after standing overnight in the ice box.

Azoproteins prepared with chicken serum:	Immune sera obtained with antigens prepared from horse serum and:			
	Para-aminobenzoic acid		Para-toluidine	
	No. 816		No. 770	
	1 hr.	Night	1 hr.	Night
Para-aminobenzoic ester azoprotein	0	tr.	+±	++
Same immediately after mixing with alkali	f.tr.	±	+±	++
Same after 5 min. hydrolysis	±	+	+±	++
Same after 1 hr. hydrolysis	+±	+±	+	+±
Same after 4 hrs. hydrolysis	+±	++	tr.	+
Same after 6 hrs. hydrolysis	++	++±	0	+
Same after 8 hrs. hydrolysis	++	+++	0	tr.
Same after 20 hrs. hydrolysis	++	+++±	0	tr.
Para-aminobenzoic acid azoprotein	++	+++±	0	tr.
Para-toluidine azoprotein	0	tr.	++	++±
Aniline azoprotein	0	f.tr.	++	++±

The experiment shows that during the course of the hydrolysis the precipitation with the serum for para-toluidine decreases gradually while correspondingly an increase in the strength of the reactions takes

In Vol. xlv, No. 6, page 1053, Table VII, last column, the third entry from the bottom should be 0 instead of tr.

place with the immune sera for para-aminobenzoic acid. The former reaction did not disappear completely but a trace of precipitate was still noticed after 20 hours hydrolysis when the tests were read on the following day.

SUMMARY.

The method of partial synthesis of antigens as employed in the foregoing experiments obviously cannot be substituted for the chemical study of natural antigens. But some questions of a rather general nature not easily accessible to investigations of the latter sort, may be approached by the use of artificial protein compounds. Thus the results reported indicate a peculiarity of certain chemical structures such as acid radicals.

The group of immune sera obtained by injecting azoproteins made from non-acid azo components had a wide range of activity. Substituents like CH_3, OCH_3, NO_2, Cl, Br, I, in the aromatic nucleus altered the reactions to a moderate degree only, in most cases.[8] The effects were dependent more on the position than on the nature of the substituents. Two substances were found, however, which had a pronounced effect on the specificity of the compound protein, namely acetyl-para-phenylenediamine and para-aminoacetophenone. In consideration of the above facts it is uncertain whether the antigenic changes noticed by Obermayer and Pick (7) after treating proteins with nitric acid, nitrous acid or iodine are mainly due to the substitution of hydrogen in the benzene ring by NO_2 and I, as is the general belief, or to other changes of the protein. This question had been raised already by the observation that the proteins treated with HNO_3 or HNO_2 containing respectively the nitro or the diazo group, did not differ substantially in their serological properties (8).

The antigens made from acid compounds form a group with distinctive features. In the first place the presence of acid radicals destroys the reactivity with the immune sera for the non-acid substances. This influence is so marked that even the reaction with the species-specific part of the protein, if such is present, appears to be diminished. Also the sera produced with the acid antigens react but feebly with the non-acid azoproteins. Accordingly it was pos-

[8] *Cf.* Wells,[1] page 79.

sible to show that by hydrolysis of the ester of an aromatic acid contained in an azoprotein the serological reactions of the antigen underwent a radical change.

The presence of a free carboxyl group in the antigens not only determines the characteristics mentioned but there is reason to believe that it increases markedly the degree of specificity exhibited by the antigens and the corresponding immune sera, when cross-tests are made with a number of acid azoproteins and their antisera. This is brought out by a comparison of the results of the present investigation with those described previously (3). It is of interest in this respect that the specific carbohydrates found by Avery and Heidelberger in pneumococci and pneumobacilli are mostly, if not in all cases, compounds of distinctly acid character.

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EXPLANATION OF PLATE 31.

FIG. 1. Reactions of immune serum for para-toluidine No. 770 with various azoproteins prepared from chicken serum and the following substances, readings after 1 hour:

1. Aniline.
2. Para-toluidine.
3. Ortho-chloroaniline.
4. Ortho-nitroaniline.
5. Meta-nitroaniline.
6. Para-nitroaniline.
7. 1, 3, 4-Xylidine.
8. 1, 2, 4-Nitrotoluidine.
9. Para-arsanilic acid.
10. Para-aminobenzoic acid.
11. Sulfanilic acid.
12. Saline control.

FIG. 2. Reactions of immune serum for para-aminobenzoic acid No. 816 with various azoproteins prepared from chicken serum and the following substances, readings after 1½ hours:

1. Aniline.
2. Para-toluidine.
3. Ortho-nitroaniline.
4. Para-nitroaniline.

5. Ortho-anisidine.
6. Para-arsanilic acid.
7. Ortho-aminobenzoic acid.
8. Meta-aminobenzoic acid.
9. Para-aminobenzoic acid.
10. Sulfanilic acid.
11. Ortho-aminocinnamic acid.
12. Saline control.

FIG. 3. Reactions of immune serum for aniline No. 760 with various azoproteins and with unchanged horse serum, reading after 15 minutes.

1. Azoprotein from chicken serum and aniline.
2. Azoprotein from horse serum and aniline.
3. Azoprotein from horse serum and para-toluidine.
4. Azoprotein from horse serum and para-nitroaniline.
5. Azoprotein from horse serum and para-aminobenzoic acid.
6. Azoprotein from horse serum and para-arsanilic acid.
7. Unchanged horse serum.
8. Saline control.

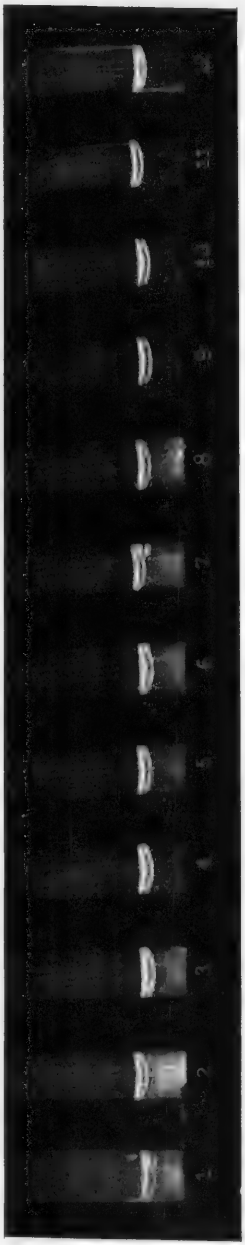

FIG. 1.

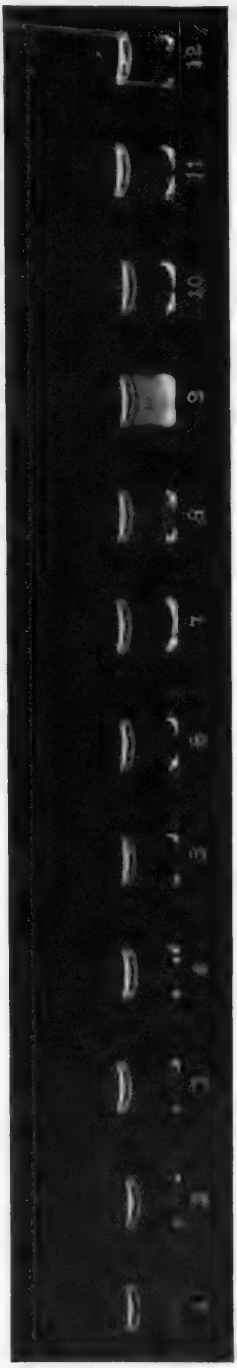

FIG. 2.

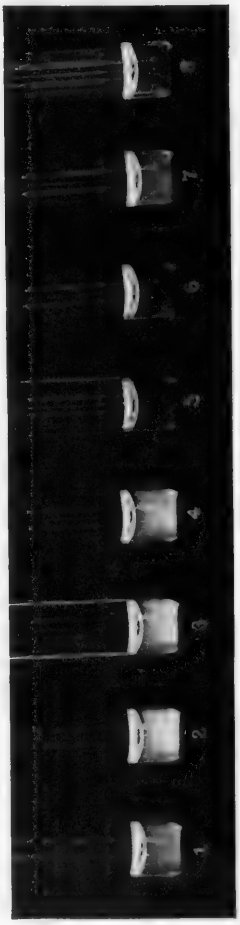

FIG. 3.

(Landsteiner and van der Scheer: Serological specificity of azoproteins.)

THE DEVELOPMENT OF AGGLUTININS AND PROTECTIVE ANTIBODIES IN RABBITS FOLLOWING INHALATION OF PNEUMOCOCCI.

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In a preceding paper (1) it has been shown that mice may acquire a high degree of active immunity following repeated inhalations of living pneumococci. However, mice are not suitable for tracing serologically the immunity developed during a series of exposures. Since rabbits may be easily and repeatedly bled, they were chosen for this work.

It has already been shown that rabbits are susceptible to infection by inhalation of Type I pneumococci (2), and that an occasional rabbit may recover from pneumococcus septicemia. In the present paper are reported the development of (1) agglutinins and (2) protective antibodies in the blood serum of rabbits following repeated inhalations of virulent Type I pneumococci. The duration of active immunity and the length of time that agglutinins and protective antibodies persist in the serum will be dealt with in a subsequent paper.

Method.

Rabbits were placed in a large spray chamber similar to that already described (3) and exposed to a spray of virulent Type I pneumococci. 50 cc. of an 8 hour broth culture were used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained from the ear vein of each animal.

The presence of agglutinins was determined by a modified thread reaction. To 1 cc. of rabbit serum diluted in normal salt solution was added 0.2 cc. of an actively growing broth culture of Pneumococcus Type I. The tubes were incubated for 2 hours in the water bath at 37°C., placed in the ice box overnight, and the reactions read the next morning. Agglutinins were recorded as present in the serum only when the reactions were positive in a dilution of at least 1:10.

The presence of protective antibodies in the blood of the sprayed rabbits was determined by the ability of 0.2 cc. of serum to protect white mice against intra-

peritoneal injection of 0.001 cc. of pneumococcus culture, of which 0.000 001 cc. killed a normal mouse within 48 hours. The rabbit serum and culture were administered simultaneously.

TABLE I.

Relation of Development of Agglutinins to the Number of Exposures.

No. of exposures	1	2	3	4	5	6	7	8	9	10
" " rabbits	135	108	87	63	48	36	34	31	25	23
" " " showing agglutinins.	4	13	12	14	12	9	9	8	6	6
Per cent of rabbits showing agglutinins	2	12	13	22	25	25	26	25	24	26

TABLE II.

First Appearance and Titre of Agglutinins.

Following exposure	1	2	3	4	5	6	7	8	9	10
Rabbit 1	—	1:10								
2	—	1:10								
3	1:50	1:50								
4	1:50	1:100								
5	—	—	—	1:20						
6	—	—		1:20						
7	1:20	1:20	1:20	1:20						
8	—	—	1:50	1:50						
9	—	1:20	1:20	1:20	1:20					
10	1:50	1:100	1:100	1:100	1:100					
11	—	1:10	1:20	1:20	1:20					
12	—	—	1:10	1:10	1:10	1:10				
13	—	—	—	—	—	—	1:10			
14	—	1:10	1:40	1:40	1:100	1:100	1:100	1:100		
15	—	—	1:10	1:20	1:100	1:100	1:100	1:100		
16	—	1:40	1:40	1:50	1:50	1:50	1:100	1:100	1:100	1:100
17	—	1:10	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50
18	—	—	—	—	1:100	1:200	1:200	1:200	1:200	1:200
19	—	1:20	1:40	1:40	1:40	1:40	1:40	1:40	1:40	1:50
20	—	—	—	—	1:10	1:10	1:20	1:40	1:40	1:40
21	—	1:20	1:40	1:40	1:50	1:50	1:50	1:50	1:50	1:50

Development of Agglutinins.

Out of 231 rabbits sprayed from 1 to 10 times, 80 or 34 per cent died from pneumococcus septicemia. The greatest number of deaths occurred after the first exposure. The sera for the serological reactions

which form the basis of this paper, were obtained from rabbits which had not only the greatest natural resistance, but also a certain degree of immunity acquired as the result of repeated exposures to live pneumococci.

In Table I are shown the number of rabbits which were sprayed from 1 to 10 times, the number which died of pneumococcus septicemia, and the number whose serum showed agglutinins in dilutions of at least 1:10. From Table I it is seen that only 4 or 2 per cent of the 135 rabbits which survived the first exposure developed demonstrable agglutinins. The percentage of rabbits showing agglutinins rose after the 2nd spray to 12 per cent, increasing to 25 per cent on and after the 5th spray. In fact 17 rabbits, after having been sprayed 10 times, failed to develop demonstrable agglutinins.

In all, 21 rabbits developed agglutinins. In Table II are shown the exposure after which agglutinins first appeared and the titre of agglutinins following each subsequent spraying. Although 4 rabbits showed agglutinins after the first exposure, a second exposure was necessary to incite the formation of these antibodies in 9 others. 1 rabbit first showed agglutinins only after 7 exposures. Great variations are also seen in the antibody titre of the different animals. As a rule agglutinins were first demonstrable only in the higher concentrations of serum (1:10 or 1:20) but in one instance the reaction first appeared following the 5th spray and was then present in a dilution of 1:100. Following the initial appearance of agglutinins there is a tendency for the titre to rise after the next spray and then to remain stationary. Of 8 rabbits which were sprayed 8 times, only 3 showed an agglutinin titre of 1:100; the serum of 3 others showed a titre as high as 1:50, while in one exceptional instance the serum suddenly showed agglutinins after the 5th spray in a dilution of 1:100 increasing after the 6th spray to 1:200.

Protective Antibodies.

In order to determine whether the serum of normal rabbits contains any natural antibodies against Type I pneumococcus, 1 cc. of serum from each of 147 normal rabbits was injected intraperitoneally into as many mice. The next day these mice were injected intraperitoneally with 0.000,001 cc. of virulent culture of Type I pneumococcus.

136 or 92 per cent of these mice died. In order to determine whether the 11 surviving mice recovered by virtue of a natural antibody present in certain normal rabbit sera, or by reason of a non-specific reaction induced by foreign protein, plain broth and normal horse serum were tested. 25 mice received in like manner injections of 1 cc. of plain broth, and another 25 were injected with 1 cc. of normal horse serum. 24 hours later all 50 mice were inoculated intraperitoneally with 0.000,001 cc. of virulent Type I pneumococcus. Of the mice receiving a preliminary injection of broth 24 or 96 per cent died, and of those injected with horse serum 17 or 68 per cent succumbed to subsequent infection. From the results of this experiment it would seem that the mere preliminary injection of a foreign serum of either the horse or rabbit, in certain instances conferred protection in mice against an

TABLE III.

Number of Rabbits Exposed and Number Whose Serum Protected Mice against 0.001 Cc. of Pneumococcus Type I.

No. of exposures	1	2	3	4	5	6	7	8	9	10
" " rabbits	135	108	87	63	48	36	34	31	25	23
" " " whose serum protected	5	10	14	25	22	22	21	23	19	19
Per cent of rabbits whose serum protected	3	9	16	39	45	61	61	74	76	82

otherwise fatal inoculation of Type I pneumococcus. From this it is evident that the 8 per cent of normal rabbit sera which afforded protection did so not because of the presence of natural antibodies but because of the non-specific protective reaction induced by the foreign serum.

In Table III are shown the number of rabbits which were sprayed from 1 to 10 times with Type I pneumococci and the number thus exposed whose serum subsequently protected mice against intraperitoneal injection of 0.0001 cc. of a virulent culture of the homologous organism. From this table it is seen that protective antibodies were demonstrable in the sera of 5 or 3 per cent of the rabbits after the 1st spray. Following each successive spraying the number of rabbits showing protection steadily increased, until after the 10th spraying the

serum of 82 per cent of the animals conferred passive protection on mice against at least 1000 lethal doses of virulent culture. A total of 49 rabbits developed protective antibodies. The spray following which the rabbit sera first protected mice is shown in Table IV.

From Table IV it is seen that with each successive spray the number of rabbits in whose sera protective antibodies were demonstrable progressively increased. Although the greatest number of rabbits showed protective antibodies in their sera after the 6th spray, other rabbits did not develop these antibodies until after the 10th exposure.

Correlation of Agglutinins and Protective Antibodies.

It is difficult to compare the relative titre of the sera at any one time because of the difference in the standards used. Whereas agglutinins were recorded as positive if present in serum concentrations of 1:10,

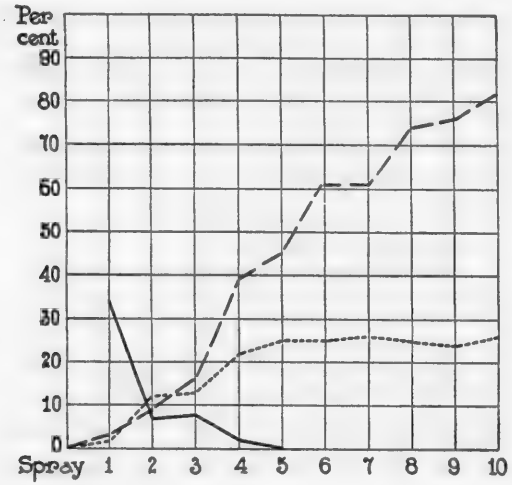
TABLE IV.

First Appearance of Protective Antibodies.

No. of spray	1	2	3	4	5	6	7	8	9	10
" " rabbits first showing protective antibodies	5	7	7	12	7	5	1	4	—	2

protective antibodies were noted only if they were present in concentrations sufficient to protect mice against a 0.0001 cc. of virulent pneumococcus culture. If smaller doses of Pneumococcus had been used, the presence of protective antibodies would undoubtedly have been detected earlier and the incidence of their occurrence would have been more frequent. However, when these antibodies were demonstrable under the conditions of this experiment, their presence was evidence of a high degree of immunity. In 10 instances both agglutinins and protective antibodies occurred after the same spray; in 9 animals agglutinins appeared before protective antibodies were demonstrable, while in 30 instances protective antibodies occurred without demonstrable agglutinins in the serum. In the 9 rabbits in which agglutinins appeared first, protective antibodies were later demonstrated in 4 instances after the next spraying, in 2 others after 3 additional sprayings, and in 1 rabbit not until after 5 subsequent exposures.

The relative occurrence of agglutinins and of protective antibodies in the serum of rabbits following inhalations of pneumococci is graphically shown in Text-fig. 1. In this figure the mortality curve of rabbits dying from septicemia is also given. It is seen that after the 1st exposure 34 per cent of rabbits died from pneumococcus septicemia. However, no rabbits died from this cause after the 4th spraying. In other words, the more highly susceptible animals were rapidly elimi-



TEXT-FIG. 1. Comparison of mortality, and presence of protective antibodies and agglutinins in rabbits following repeated inhalations of virulent Type I pneumococci.

———— per cent of rabbits dying with pneumococcus septicemia following successive sprayings.

- - - - - - - per cent of rabbits showing agglutinins in serum following spraying.

═══ per cent of rabbits showing protective antibodies in their serum.

nated by the 1st exposure, while those that survived were either naturally more resistant or had gained some degree of immunity. It is interesting to note that up to the 5th spray an increasing proportion of rabbits developed agglutinins but that after this the percentage remained stationary. On the other hand, with each successive spray an increasing number of rabbits developed protective antibodies until

following the 10th exposure 82 per cent of the total number showed the presence of these immune substances in their serum.

DISCUSSION.

From the foregoing experiments it appears that following repeated inhalations of living Type I pneumococci, rabbits develop a high degree of immunity as evidenced by the presence of demonstrable agglutinins and protective antibodies in their serum. This immunity is probably induced by a few organisms penetrating the respiratory epithelium and entering into the body tissues. It has already been shown that rabbits may even recover from a transient pneumococcus septicemia. The great variations both in the time of first appearance and in the final titre of immune bodies are difficult to explain.

Among the factors which cannot be experimentally controlled are: first, the number of organisms which come to lodge within the respiratory tract following exposure to a bacterial spray; second, the number which after implantation are able to invade the tissues, and third, the final disposition of these bacteria in the animal body. In certain instances the multiplication of the invading organisms goes on unchecked until the death of the animal. In others a transient carrier state may occur with subsequent immunity responses. It is certainly significant that whereas the curve of incidence of protective antibodies steadily increases, the percentage of rabbits showing agglutinins does not materially change after the 5th spraying.

CONCLUSIONS.

1. Following repeated inhalations of Type I pneumococci agglutinins and protective antibodies can be demonstrated in the serum of rabbits.

2. The percentage of rabbits whose serum shows agglutinins remains stationary after the 5th exposure, but the percentage of rabbits showing protective antibodies in their sera steadily rises.

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THE RESPONSE TO RESPIRATORY RESISTANCE.

A Comparison of the Effects Produced by Partial Obstruction in the Inspiratory and Expiratory Phases of Respiration.

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The liability to fatigue of the respiratory center is a subject which needs to be studied. Davies, Haldane, and Priestley (1) were the first to investigate the manner in which breathing adapts itself to increased resistance, and the point at which the adaptation begins to fail. They showed that the normal response in man to respiratory resistance in both phases of respiration is slow and deep breathing. When the resistance is excessive respirations become progressively shallower and more frequent and the subjects then become cyanotic. Haldane and his coworkers believe that this is due to fatigue of the respiratory center. They believe anoxemia hastens greatly the onset of fatigue and the ease with which it is produced. They conclude that the mechanism involved in the immediate response is the Hering-Breuer reflex, pointing out that as a result of resistance, the time required for inflation or deflation of the lungs to reach the point at which the Hering-Breuer stimulus becomes effective is prolonged, that CO_2 accumulates in the meantime, and that the next respiration is deep and vigorous. The more or less sudden onset of rapid, shallow breathing Haldane interprets as evidence of fatigue of the respiratory center, with a resulting predominance of the peripheral stimuli over the central impulses normally governing breathing.

A study of these effects in animals was undertaken by us with several points in mind. We hoped for additional information as to the nature and origin of rapid and shallow breathing, which we have previously considered in both clinical and experimental studies (2–5). It seemed highly desirable to learn something about the liability to fatigue of so vital a structure as the respiratory center. Transferring the problem

to experimental animals rather than man, though it introduced such complications as the use of anesthetics, afforded the opportunity of allowing the experiments to go to their natural conclusion. It made it possible, too, to study the end-results of more or less prolonged periods of anoxemia and rapid and shallow breathing. It was soon learned that the response to resistance in inspiration is strikingly different from the response to resistance in expiration, both as regards functional and structural changes.

Method.

Dogs anesthetized with barbital-sodium were used. The dogs varied in weight from 6.8 to 23.5 kilos, but in every instance except one the weight of the animal was above 10 kilos. The barbital-sodium was dissolved in physiological sodium chloride solution and given intravenously in an amount sufficient to produce complete relaxation and a slow, steady respiratory rate. The initial dose was calculated on a basis of 0.3 gm. drug to 1 kilo body weight and the additional drug was given in repeated small quantities as necessary.[1] The rectal temperature was recorded at frequent intervals throughout the course of each experiment. As a precaution against the loss of heat each animal was wrapped snugly in woolen blankets and surrounded by warm air.[2]

When the animal had reached the desired state of anesthesia, tracheotomy was done, and a properly fitting rubber tube was tied firmly into the trachea. This tube, which was of such a length as not to increase the dog's natural dead space, communicated with one arm of a four-way metal tube. Two of the other branches of the four-way tube were connected by corrugated tubing of the usual type used in respiration experiments to inspiratory and expiratory valves. Low resistance valves of the kind recently described by Dr. C. V. Bailey[3] (6) were used in all experiments.

The fourth opening in the metal tube was connected to a rubber tambour by means of a short length of rubber pressure tubing. This tambour, moving with expiration and inspiration, activated a make and break contact in an electrical circuit which included an electromagnet "telephone" counter. By this arrangement the respiratory rate was automatically counted. Intratracheal pressure was measured by a water manometer communicating by means of a Y-tube with the

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[1] A 5 per cent solution was used for this purpose.

[2] A cradle made by covering an arch of thin metal with hair felt was placed over the animal. Heat was supplied by an electric bulb suspended from the top of the arch. We have found that this is a much more efficacious way of maintaining the warmth of an animal than by use of an electric pad.

[3] These valves were kindly supplied us by Dr. Bailey.

four-way tracheal tube. The expired air was collected in a large Tissot spirometer. To introduce resistances we used a specially constructed metal tap with an

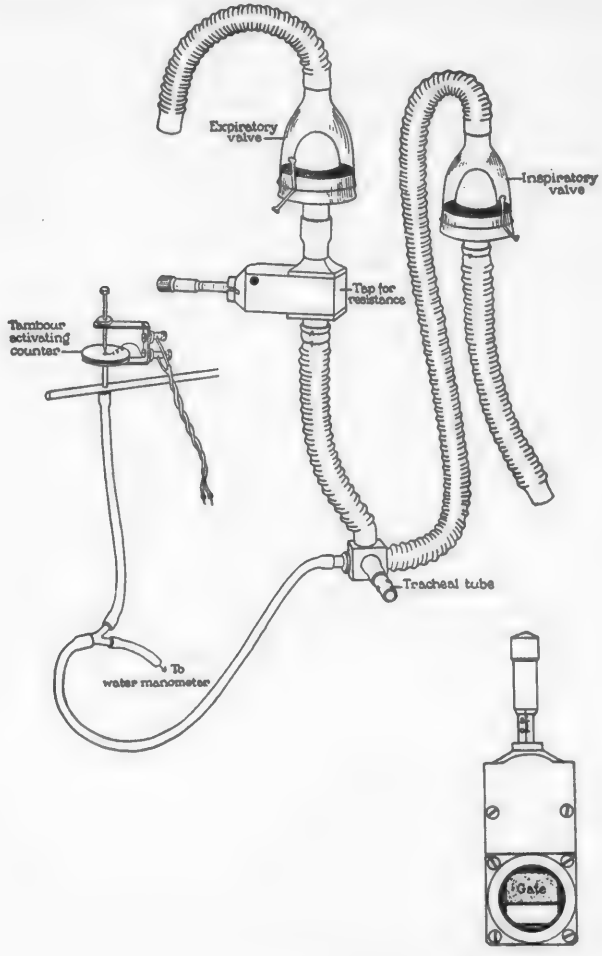

FIG. 1.

internal bore of 4 sq. cm. The tap was closed by means of a gate which was gradually lowered into its seat by a screw of such a pitch that one turn lowered the gate 1 mm. and accordingly diminished the lumen by 0.2 sq. cm. A scale permitted one

to read off at any time the cross-section area in sq. cm. This tap was introduced into the system at a point between the trachea and one of the respiratory valves. According to its position, resistance could be created either in the inspiratory or expiratory phases of respiration. The apparatus which we used is shown in diagram in Fig. 1.

When resistance to respiration is introduced by a tap of this sort the velocity of air flow varies as the square root of the driving pressure. With cotton wool resistance, however, which Haldane and his coworkers used, the air flow varies directly as the driving pressure. The intratracheal pressure may be assumed to represent the driving pressure. Velocity of air flow can thus be calculated if desired. We have chosen tap resistance because it is easier of manipulation and quantitative control. It was found that great reduction in the cross-section area of the tap was necessary before any change in type of breathing occurred.

All experiments were conducted on the same general plan and the observations fall into three periods; first, the period of control; second, the period of resistance; and, third, the period following release.

The observations include a record of respiratory rate, tidal air, minute volume of pulmonary ventilation, intratracheal pressure, cross-section area of the tap, and in some instances an additional study of the O_2 content, O_2 capacity, percentage oxygen saturation, CO_2 content, CO_2 tension, and pH of the arterial blood. The blood samples were withdrawn through a cannula in the femoral artery and collected without exposure to air in sampling tubes over mercury. The analyses of oxygen content and capacity, and of CO_2 content of the separated plasma or serum were made by the method of Van Slyke and Neill (7). The pH was estimated on serum by the colorimetric method of Hastings and Sendroy (8). From these data and the following formula (9) the partial pressure of carbon dioxide expressed in mm. Hg was calculated, assuming pK' to be 6.115.

$$pCO_2 = \frac{[CO_2]}{0.031 \times (1 + 10^{pH - 6.115})}$$

where CO_2 content is given in terms of millimols per liter.

The records of respiratory rate and pulmonary ventilation were made over 5 minute periods, and were repeated several times under each condition. During the time of resistance the cross-section area of the tap was reduced to a degree that created a negative or positive intratracheal pressure of from 10 to 20 cm. of H_2O, and this was maintained over intervals ranging from 20 to 145 minutes. The readings in the final period were taken when the respiratory rate had reached a constant, or approximately constant, level.

In some experiments the animals breathed room air. In others they breathed 90 to 95 per cent oxygen from a Douglas bag. After the final observations the experiment was terminated by the intravenous injection of from 20 to 30 cc. of a saturated solution of magnesium sulfate. At autopsy attention was given to the presence or absence of froth in the trachea, pleural effusions and gross edema of

the mediastinal tissues and lungs, and to the color of the lungs. The degree of hypostatic congestion, the lung weights, the heart weights, and the lung-heart ratio were also observed.

TABLE I.

Experiment 7. The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	*per cent*
Control period	11.57 to 12.46	Room air	19	273	5.11	25.8	32.91	7.50	8.47	9.02	93.9
During resistance	12.46 to 2.55	Room air	30	92	2.78	28.1	53.15	7.32	6.29	9.39	67.0
After release	2.55 to 3.48	Room air	40	225	9.00	24.5	28.63	7.54	8.89	9.66	92.0

Weight of animal, 23.5 kilos.
Total barbital-sodium, 0.35 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.05 sq. cm.
Negative intratracheal pressure, 20 cm. H_2O.
Duration of greatest resistance, 113 min.
Lung-heart ratio, 1.66.

EXPERIMENTS.

I. Experiments with Resistance in the Inspiratory Phase.

Five experiments were performed in which the cross-section of the tap opening was 0.05 sq. cm. In these experiments the negative intratracheal pressure during the inspiratory phase varied from 11 to 20 cm. of H_2O. In a sixth experiment a conspicuous effect on breathing was obtained with a cross-section area of 0.1 sq. cm. All animals showed an increase in respiratory rate and a decrease in tidal air. The percentage increase in respiratory rate ranged from a low extreme of 58 per cent to a high extreme of 310 per cent, and the percentage

decrease in tidal air ranged from a high extreme of 88.9 per cent to a low extreme of 56.1 per cent. The experimental data are presented in Tables I to IV.

Examination of Table I will bring out the following points. A dog anesthetized with barbital-sodium was breathing at the rate of 19 per

TABLE II.

Experiment 1A. The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	*per cent*
Control period	12.47 to 1.35	90–95 per cent O_2	15	189	2.73	26.29	41.8	7.40	9.46	8.77	107.8
During resistance	1.35 to 3.04	90–95 per cent O_2	47	21	1.00	32.08	80.2	7.19	5.27	10.44	50.4
After release	3.04 to 3.24	90–95 per cent O_2	139	75	10.4	25.61	49.7	7.31	5.75	10.66	54.0

Weight of animal, 14 kilos.
Total barbital-sodium, 0.37 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.1 sq. cm.
Negative intratracheal pressure not recorded.
Duration of greatest resistance, 19 min.
Lung-heart ratio, not determined.

minute. The caliber of the tap was gradually reduced until it had reached 0.05 sq. cm. This was sufficient to cause a negative intratracheal pressure at the height of inspiration equal to 20 cm. H_2O. Under these conditions the rate accelerated to 30. This was accompanied by a decrease in tidal air from 273 cc. to 92 cc. and a resulting

decrease in minute volume of pulmonary ventilation from 5.11 liters to 2.78 liters. These changes occurred while the animal was breathing room air. They were associated with a rise in pCO_2 from 32.91 mm. to 53.15 mm., a drop in pH from 7.50 to 7.32, and a decrease in the percentage oxygen saturation of the arterial blood from 93.9 to 67.0. Resistance was maintained for 113 minutes. 65 minutes after the

TABLE III.

Experiment 5. The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	*per cent*
Control period	12.35 to 1.31	Room air	8	249	1.98	25.26	52.05	7.28	9.37	10.34	90.6
During resistance	1.31 to 3.49	Room air	17	80	1.36	29.40	61.95	7.27	6.00	10.48	57.3
After resistance	3.49 to 4.07	Room air	8	285	2.28	25.75	53.05	7.28	9.17	10.01	91.6

Weight of animal, 17.25 kilos.
Total barbital-sodium, 0.32 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.05 sq. cm.
Negative intratracheal pressure, 11.5 cm. H_2O.
Duration of greatest resistance, 124 min.
Lung-heart ratio, 1.71.

valve was opened the respiratory rate was 40, the tidal air was 225 cc., and the minute volume was 9 liters. The pCO_2 had dropped to 28.63 mm., the pH had risen to 7.54, and the percentage oxygen saturation of the arterial blood was again normal, 92 per cent. Postmortem examination showed no froth in the trachea, no effusion into either pleural cavity, and no edema of the mediastinal tissues. The surface of the lungs appeared granular; the color was a bright pink. Hy-

postasis was limited to the dependent parts of the lobes. The lung-heart ratio was 1.66.

In Experiment 1A (Table II) the animal breathed 90 to 95 per cent oxygen. In spite of this, after inspiring against resistance for 21 minutes the percentage oxygen saturation of the arterial blood dropped from 107.8 to 50.4. The rate accelerated from 15 to 47 and the tidal air dropped from 189 cc. to 21 cc. The tension of CO_2 rose from 41.8 mm. to 80.5 mm. and the pH decreased from 7.40 to 7.19. The final estimations in this animal were made 34 minutes after the resistance was removed. It should be noted that the respiratory rate at this time was 139, the tidal air only 75 cc., the tension of carbon dioxide still above the original level, and the percentage oxygen saturation of the arterial blood only 54. Autopsy showed a considerable amount of frothy liquid in the trachea and 10 to 15 cc. of clear fluid in each pleural cavity. The lungs were boggy with fluid.

In Experiment 5 (Table III) the increase in CO_2 tension during the period of resistance was slight, and the pH did not change. The animal breathed air, and the percentage oxygen saturation of the arterial blood dropped from 90.6 to 57.3. This accompanied an increase in respiratory rate from 8 to 17 and a decrease in tidal air from 249 cc. to 80 cc. Resistance was continued 124 minutes. 41 minutes after release all functions were at the normal level. Autopsy in this case showed no froth in the trachea, no effusion into either pleural cavity, and no edema of the mediastinal tissues. The lungs were diffusely coral-pink in color. Hypostatic congestion was slight. The lung-heart ratio was 1.7.

The data for Experiments 2, 3, and 4 are grouped together in Table IV. The results in general are consistent with those obtained in Experiments 1A and 7. The increase in minute volume during the period of resistance in Experiment 3 was a single occurrence. In Experiments 2 and 3 the animals breathed 90 to 95 per cent oxygen and maintained a pink color of the tongue and mucous membranes throughout. It seems safe to conclude that resistance in these animals did not produce anoxemia. At autopsy pleural effusions were found in Experiments 3 and 4. In Experiment 3 there was in addition an excessive edema of the mediastinal tissues. The color of the lungs in all cases was a coral-pink, of varying shades of intensity, but deeper in every instance

TABLE IV.

Experiments 2, 3, and 4.

The Effect of Resistance in the Inspiratory Phase of Respiration.

Conditions	Experiment 2					Experiment 3					Experiment 4				
	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume
				cc.	*liters*				*cc.*	*liters*				*cc.*	*liters*
Control period	2.27 to 2.41	90–95 per cent O_2	18	114	2.09	11.01 to 11.22	90–95 per cent O_2	10	184	1.84	12.03 to 1.29	Room air	19	203	3.86
During resistance	2.41 to 5.15	90–95 per cent O_2	35	50	1.76	11.22 to 2.18	90–95 per cent O_2	41	64	2.62	1.29 to 4.30	Room air	39	80	3.24
After release	5.15 to 6.04	90–95 per cent O_2	44	90	3.98	2.18 to 3.40	90–95 per cent O_2	118	105	12.42	4.30 to 5.14	Room air	34	172	5.84

Weight of animal, 6.8 kilos.
Total barbital-sodium, 0.46 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.05 sq. cm.
Negative intratracheal pressure, 14.5 cm. H_2O.
Duration of greatest resistance, 38 min.
Lung-heart ratio, 1.75.

Weight of animal, 17 kilos.
Total barbital-sodium, 0.30 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.05 sq. cm.
Negative intratracheal pressure, 15.5 cm. H_2O.
Duration of greatest resistance, 145 min.
Lung-heart ratio, 2.12.

Weight of animal, 16.5 kilos.
Total barbital-sodium, 0.33 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.05 sq. cm.
Negative intratracheal pressure, 15 cm. H_2O.
Duration of greatest resistance, 70 min.
Lung-heart ratio, 1.53.

than the shade of a normal lung. Hypostatic congestion in Experiments 3 and 4 was moderate. The lungs in Experiment 2 were completely free of hypostasis.

The results of these experiments may be summed up as follows: Resistance to inspiration results in a fall in intratracheal pressure which is associated with an increase in respiratory rate and a decrease in tidal air. In most instances these are accompanied by a severe limitation of the minute volume of pulmonary ventilation. The effects come on suddenly when the cross-section area of the inspiratory passageway is reduced to 0.1 sq. cm. or 0.05 sq. cm. Anoxemia accompanies these changes, but may be prevented by the inhalation of 90 to 95 per cent oxygen. Associated with the anoxemia there is a retention of carbon dioxide and usually a drop in pH.

When resistance is removed the respiratory rate continues to be rapid. In the majority of instances the rates were higher following release than they were during the period of resistance. Release, however, permits an increase in tidal air and minute volume, and as a result of this, there is a fall in pCO_2, a rise in pH, and in some cases a complete disappearance of anoxemia. On only one occasion when resistance was removed did the respiratory rate return to the control level, with a corresponding change in CO_2 tension and oxygen saturation. The postmortem picture found in these dogs may be characterized by congestion and edema of the lungs.

II. Experiments with Resistance in the Expiratory Phase.

In striking contrast to the experiments with resistance in the inspiratory phase, partial obstruction to expiration slows the respiratory rate. This occurs with varying effects on tidal air, but with a constant decrease in minute volume of pulmonary ventilation. The results of four experiments are presented in Tables V to VII.

In Experiment 11 (Table V) the dog breathed at the rate of 25 before resistance was introduced in expiration. In the presence of restriction equal to 0.1 sq. cm. the rate dropped to 12 and the tidal air increased from 85 cc. to 122 cc. This was accompanied by a decrease in minute volume from 2.12 liters to 1.46 liters with only slight changes in pH and blood gases. The animal exhaled against resistance for 96 minutes. 24 minutes after the tap was opened the

respiratory rate had returned to the original level—thus distinguishing this type of experiment from the ones in which resistance was introduced into inspiration. No other conspicuous changes occurred. The data are given in Table V.

In Experiment 12 (Table VI) a drop in the respiratory rate from 22 to 11 was accompanied by a decrease in tidal air from 70 cc. to 49

TABLE V.

Experiment 11. The Effect of Resistance in the Expiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc.	*liters*	*mM*	*mm Hg*		*mM*	*mM*	*per cent*
Control period	12.08 to 12.39	Room air	25	85	2.12	26.62	45.15	7.37	6.35	6.68	95.1
During resistance	12.39 to 2.30	Room air	12	122	1.46	25.87	40.23	7.41	6.69	7.15	93.6
After release	2.30 to 3.12	Room air	24	110	2.64	24.51	35.72	7.44	6.24	6.59	94.7

Weight of animal, 10.5 kilos.
Total barbital-sodium, 0.32 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.1 sq. cm.
Positive intratracheal pressure, 13 cm. H_2O.
Duration of greatest resistance, 96 min.
Lung-heart ratio, 1.51.

cc. and a decrease in minute volume from 1.56 liters to 0.48 liter. The animal breathed room air, and, as could be anticipated from the great reduction in pulmonary ventilation, developed a severe anoxemia. The CO_2 content of the serum rose from 29.13 mM to 32.48 mM, the pCO_2 from 51.1 mm. Hg to 70.0 mm., and the pH dropped from 7.36 to 7.26. The period of resistance was 96 minutes. When the resistance was removed the respiratory rate returned to within five

breaths of the control level. Coincident with the recovery in rate there was an increase in tidal air and minute volume, with the result that the anoxemia was completely relieved and the pCO_2 and pH returned to their previous values.

In Experiments 1B and 10 (Table VII) there was likewise a noticeable decrease in respiratory rate and minute volume.

TABLE VI.

Experiment 12. The Effect of Resistance in the Expiratory Phase of Respiration.

Conditions	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	CO_2	pCO_2	pH	Arterial blood		
									O_2 content	O_2 capacity	Saturation
				cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	*per cent*
Control period	12.40 to 1.14	Room air	22	70	1.56	29.13	51.1	7.36	7.01	7.53	93.1
During resistance	1.14 to 2.50	Room air	11	49	0.48	32.48	70.00	7.26	4.41	8.18	53.9
After release	2.50 to 3.31	Room air	17	84	1.42	28.71	48.23	7.38	7.43	7.70	96.5

Weight of animal, 11.5 kilos.
Total barbital-sodium, 0.30 gm. per kilo body weight.
Cross-section area of tap finally reduced to 0.1 sq. cm.
Positive intratracheal pressure, 12 cm. H_2O.
Duration of greatest resistance, 96 min.
Lung-heart ratio, 1.14.

The postmortem examinations in this group of experiments brought out the following points: None of the animals showed pleural effusions, frothy fluid in the trachea, edema of the mediastinal tissues, or gross edema of the lungs. The color of the lung surface in all instances was a diffuse coral-pink, with the same slight variations in intensity as were noted in the previous series. Perhaps the most striking observation was a complete absence of hypostatic congestion in Ex-

TABLE VII.

Experiments 1B and 10. The Effect of Resistance in the Expiratory Phase of Respiration.

Conditions	Experiment 1B					Experiment 10				
	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume	Time	Gas inspired	Respiratory rate per min.	Tidal air	Minute volume
				cc.	*liters*				*cc.*	*liters*
Control period	3.15 to 3.43	90–95 per cent O_2	12	166	2.03	1.40 to 2.22	Room air	16	162	2.53
During resistance	3.43 to 6.07	90–95 per cent O_2	9	184	1.59	2.22 to 4.10	Room air	9	138	1.24
After release	6.07 to 6.26	90–95 per cent O_2	8	200	1.68		Room air	—*	—	—
	Weight of animal, 13.5 kilos. Total barbital-sodium, 0.32 gm. per kilo body weight. Cross-section area of tap finally reduced to 0.1 sq. cm. Positive intratracheal pressure not recorded. Duration of greatest resistance, 20 min. Lung-heart ratio, not determined.					Weight of animal, 11 kilos. Total barbital-sodium, 0.37 gm. per kilo body weight. Cross-section area of tap finally reduced to 0.1 sq. cm. Positive intratracheal pressure, 10 cm. H_2O. Duration of greatest resistance, 74 min. Lung-heart ratio, 1.04. * The resistance was not released in this experiment.				

periments 1B and 12, with only a slight degree of hypostasis in Experiments 10 and 11. The lung-heart ratios, calculated in three out of the four instances (Experiments 10, 11, and 12) were 1.04, 1.51, and 1.14, respectively. All of these figures are lower than those

obtained with resistance in the inspiratory phase, though at times the difference is very slight.

These experiments may be summarized thus: Resistance to expiration slows the respiratory rate and limits the minute volume of pulmonary ventilation. These changes may or may not be accompanied by a retention of carbon dioxide and a low oxygen saturation of the arterial blood. The effects come on suddenly when the cross-section area of the tap is reduced to 0.1 sq. cm. With removal of resistance all functions return to their normal, or approximately normal, levels. No characteristic pulmonary pathology occurs as the result of resistance to expiration.

DISCUSSION.

The second group of experiments is easier of interpretation than the first. With resistance to expiration there is a mechanical limitation to pulmonary ventilation which may result in carbon dioxide retention and insufficient oxygenation of the blood in the lungs. When the mechanical limitation is removed both the breathing and the state of the blood return to normal and no apparent damage to the lungs has occurred.

The interpretation of the first group of experiments, those dealing with resistance to inspiration, is not so clear. Here, too, we have a mechanical limitation to pulmonary ventilation, but the result in this case is rapid and shallow breathing rather than slow and shallow. There is, as in the other group, inadequate ventilation of the blood. But, even after the resistance had been removed, in many experiments the respiratory rate continued to be rapid, and in some this was true even though the blood had returned to the state found during the control period.

What has happened to the animal which causes it to maintain a rapid respiratory rate in spite of the fact that no longer is there any resistance to the free passage of air into the lungs? The explanation must be sought for in alterations to one of the organ systems having to do with respiratory rhythm. Has the respiratory center itself been damaged or fatigued? There does not appear to be any direct evidence for this assumption. The more or less prolonged period of anoxemia and acidosis which existed in the inspiratory ex-

periments was present in one of the expiratory experiments as well, and yet in this animal there was no accelerated rate after release of resistance. Have the muscles which have to do with inspiration become fatigued as the result of resistance? This hardly seems to be a plausible explanation because in none of the experiments was there a fall in the negative pressure produced in the trachea. Such a fall would have suggested a lessened effort at expansion of the lungs.

The explanation may be sought for more probably in the state of the lungs themselves. The decreased expansion may give rise to a state of pulmonary congestion and this, together with the heightened negative pressure, may result in a seepage of fluid into the pulmonary parenchyma and pleural sacs, as has been suggested by Graham (10). Indeed, it has been experimentally shown by Huggett (11) that inspiratory obstruction increases the minute and stroke volumes of the heart, while expiratory obstruction produces a reverse effect. An augmented blood flow through the lungs may be responsible for the congestion and fluid transudation which were actually observed. It was not unlike that seen in dogs with multiple experimental emboli of the pulmonary capillaries and arterioles (2), nor unlike the changes found after clamping and releasing the artery to one lung (4). In these conditions, too, persistent rapid and shallow breathing occurred.

The local changes in the lung may then perhaps be regarded as responsible for this phenomenon. The normal Hering-Breuer stimuli are increased and predominate. Whether this in itself is evidence of fatigue of the respiratory center, as Haldane believes, is a matter for conjecture.

SUMMARY AND CONCLUSIONS.

1. A study has been made of the effects of resistance to respiration in the inspiratory and expiratory phases.

2. Resistance to inspiration caused an increase in respiratory rate, a decrease in tidal air, and in most instances a severe limitation of the minute volume of pulmonary ventilation. Anoxemia and acidosis accompanied these changes.

3. When resistance was removed the respiratory rate continued to be rapid, but the tidal air and minute volume increased. As a result of this there was a fall in pCO_2, a rise in pH, and in some cases a complete disappearance of anoxemia.

3. Resistance to expiration slowed the respiratory rate and produced a constant decrease in the minute volume of pulmonary ventilation. Anoxemia and carbon dioxide retention occurred, but were less pronounced than in the inspiratory experiments. Release of resistance to expiration resulted in a return of all functions to their normal, or approximately normal, levels.

4. A difference in the gross pulmonary pathology found at autopsy in these two types of experiments has been described, and an attempt has been made to correlate changes in function with changes in structure.

5. No direct evidence has been supplied for the liability to fatigue of the respiratory center.

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A FURTHER STUDY OF BLOOD REACTION AND BLOOD GASES IN PNEUMONIA.

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Three years ago two of us with Neill and Morgan (1) attempted to determine whether or not a condition of acidosis exists in pneumonia. The hydrogen ion concentration, carbon dioxide content and tension, and the oxygen content and capacity were estimated in the blood of sixteen patients. In all, thirty observations were made. No tendency towards an acidosis of either metabolic or respiratory origin was observed. Indeed, the alkali reserve was within normal limits in every case. The pH, too, was in each instance within normal limits, 7.30 to 7.50, the greater number of observations falling in the more alkaline half of this range. The results, as are those of this study, were believed to contraindicate alkali therapy.

In spite of the consistency of the findings in this first series, it was thought advisable to extend the observations to a larger number of patients, and particularly to make repeated successive estimations on the blood of a few individuals. A patient suffering from pneumonia may present such a rapid succession of changes with respect to his hematorespiratory function, that he can scarcely be said to be in a steady state. What is true of him one day may not be true of him the next.

This fact, together with the changes induced by such environmental factors as serum therapy and oxygen inhalation, naturally gives one pause in generalizing about the state of the blood in so inconstant a condition as pneumonia. The results of the present study are, however, corroborative of the earlier one.

Material and Methods.

The observations reported were made on adult patients of both sexes, all suffering from pneumonia. Nineteen individuals comprise the series on whom

TABLE I.

Case No.	Observation No.	History No.	Date	Organism	Area of pulmonary involvement	Day disease blood was drawn	Day disease temperature fell to normal	Day disease patient died	pH at body temperature	CO_2 content per liter	CO_2 tension	O_2 content per liter	O_2 capacity per liter	Saturation	Temperature	Pulse	Respiration	Breathing room air	Breathing O_2. O_2 in chamber	Treatment	Remarks
										mM	*mm. Hg*	*mM*	*mM*	*per cent*	*°C.*				*per cent*		
1	1	5230	2– 2–25, a.m.	Type I	R. U. L.	6			7.30	21.52	52.4	6.02	8.18	73.6	101.4	118	40	Yes		Type I serum	Empyema Surgical drainage
	2		2– 2–25, p.m.		R. U. L.	6			7.48	21.20	36.0	7.75	8.51	91.1	100.4	102	40		39	Type I serum	Suppurative pleurisy and pericarditis
	3		2– 5–25, a.m.		Empyema	9			7.45	25.80	44.8	6.74	6.93	97.3	100.4	98	40		38	Type I serum	Death
	4		2– 5–25, p.m.		Empyema	9			7.50	25.22	39.1	5.73	6.95	82.5	101.4	96	36	Yes			
	5		2– 6–25		Empyema	10		42	7.47	24.52	40.7	5.95	7.01	84.9	101.4	104	36	Yes		Transferred for operation	
2	6	5233	2– 4–25	Grp. IV	R. U. L.	3			7.53	22.03	33.1	7.59	7.80	97.3	100.4	125	24	Yes			Recovery
	7		2– 6–25		R. U. L.	5	6		7.38	24.92	51.9	7.30	8.51	85.9	100.4	88	32	Yes			
3	8	5253	2–20–25	Type I	R. U. L.	3		4	7.42	22.45	45.3	8.13	9.36	86.8	101	120	32		38	Type I serum	Bilateral serofibrinous pleurisy. Death

4	9	5257	2–21–25, a.m.	Grp. IV Staph.	R. L. L.	2			7.47	22.38		6.65		68.3	100.4	120	40	Yes			Bilateral serofibrinous pleurisy. Fibrinous pericarditis. Death
	10		p.m.		R. L. L.	2		3	7.50	22.56	37.6	7.42	9.69	76.6	100.4	130	36		38		
5	11	5259	2–23–25	Grp. IV	L. L. L.	2			7.40	20.55	41.3	5.73	8.04	71.3	100.4	116	38	Yes			Death
	12		2–24–25		L. L. L.	3		3	7.45	19.95	36.3	6.44	8.51	75.7	103.4	130	36	Yes			
6	13	5268	3– 2–25	Grp. IV	R. U. L.	4			7.46	22.96	38.9	5.73	6.82	84.0	102.1	76	31	Yes			Recovery
	14		3– 3–25			5			7.49	24.20	38.3	6.01	6.53	92.0	101.6	72	30		39		
	15		3– 4–25			6			7.49	24.86	39.1	6.15	6.32	97.3	101	80	34		41		
	16		3– 5–25			7	7		7.40	26.70	50.2	5.90	6.40	92.2	98.6	64	28		39		
	17		3– 6–25			8			7.48	25.60	40.1	5.70	6.01	94.9	97.6	60	22	Yes			
	18		3– 7–25			9			7.44	24.28	41.9	5.94	6.52	91.1	97.8	68	24	Yes			
	19		3– 9–25			11			7.34	19.69	42.1	6.31	6.58	95.9	98.4	68	22	Yes			
	20		3–10–25			12			7.45	20.27	34.8	6.32	6.86	92.2	98.6	60	25	Yes			
	21		3–13–25			15			7.37	21.74	43.6	6.40	6.64	96.4	98.2	76	20	Yes			
7	22	5278	3– 8–25	Grp. IV	R. L. L.	4			7.41	23.08	43.9	6.31	6.93	91.1	103	108	28	Yes			Recovery
	23		3–10–25			6			7.44	23.31	41.4	6.28	7.16	87.7	101.3	108	24	Yes			
	24		3–11–25			7			7.46	23.58	41.7	6.29	6.71	93.8	100.4	88	32	Yes			
8	25	5292	3–19–25	Grp. IV	L. L. L.	3	11		7.40	20.59	41.5	7.56	7.95	95.1	103.6	104	28	Yes			Recovery
9	26	5300	3–23–25	Type I	L. L. L. R. U. L.	4			7.37	25.60	53.4	4.92	7.67	64.2	102	110	48	Yes		Type I antipneumococcus serum	Empyema. Surgical drainage. Recovery
	27		3–24–25			5			7.33	26.82	60.9	7.15	7.63	93.7	101	92	36		40		
	28		3–25–25			6	9		7.43	23.73	43.7	6.68	7.83	85.4	100.4	72	34	Yes			

TABLE I—*Concluded.*

Case No.	Observation No.	History No.	Date	Organism	Area of pulmonary involvement	Day disease blood was drawn	Day disease temperature fell to normal	Day disease patient died	pH at body temperature	CO_2 content per liter	CO_2 tension	O_2 content per liter	O_2 capacity per liter	Saturation	Temperature	Pulse	Respiration	Breathing room air	Breathing O_2. O_2 in chamber	Treatment	Remarks
										mM	*mm. Hg*	mM	mM	*per cent*	°C.				*per cent*		
10	29	5310	3–27–25	Type I	R. M. L. R. L. L.	5			7.38	24.30	52.7	7.56	9.76	77.5	103.2	106	32	Yes		Type I anti-pneumococcus serum	Death
	30		3–28–25			6			7.49	23.41	39.8	7.69	8.84	87.0	104	118	50		39		
	31		3–31–25			9		10	7.42	29.61	52.1	3.55	4.98	71.3	101.8	138	56		38		
11	32	5314	3–30–25, a.m.	Type III	L. L. L.	7			7.33	20.70	44.8	3.55	5.68	62.5	102.8	86	32	Yes			Recovery
	33		3–30–25, p.m.				13		7.49	21.80	33.3	4.26	5.33	80.0	102	84	50		50		
12	34	5317	4– 6–25	*Staph. aureus*	R. L. L. R. M. L.	7			7.50	21.55	32.1	4.88	5.37	90.9	101.2	100	28		40		Empyema Surgical drainage. Recovery
13	35	5347	5–18–25, a.m.	Type III	L. U. L. L. L. L.	7			7.34	21.95	50.1	6.47	9.07	71.3	100.6	124	32	Yes			Septicemia (Type III).
	36		5–18–25, p.m.			7				23.55		8.80	8.97	98.1	98.2	112	32		50		Chronic morphine poisoning. Recovery
	37		5–23–25			12			7.48	27.40	45.5	6.19	8.02	77.2	99.2	104	36	Yes			
	38		5–25–25			14			7.41	28.44	54.2	7.20	7.64	94.3	98.8	76	22		40		
	39		6– 2–25			15				27.20		5.24	7.96	65.8	100.4	90	28	Yes			
	40		5–26–25			22	7		7.44	32.55	6.25	5.53	6.43	86.0	98.6	73	16	Yes			

14	41	5470	12–15–25	Type III	L. L. L.	4	5		7.47	25.88	41.8	5.54	5.74	96.5	102.2	100	32	Yes			Recovery
15	42	5472	12–21–25	Grp. IV	R. U. L. R. L. L.	7	12		7.38	25.76	48.6	2.74	4.41	62.1	102	120	42	Yes			Recovery
16	43	5476	12–19–25	?	L. L. L.	5	11		7.37			4.99	5.22	95.6	104	72	25	Yes			Recovery
17	44	5490	12–30–25, a.m.	Type I	L. U. L. L. L. L.	4			7.50	32.62	49.1	3.02	5.48	55.1	102	130	38	Yes		Type I serum. Transferred for operation	Empyema. Surgical drainage. Recovery
	45		12–30–25, p.m.				12		7.46			4.65	5.29	87.9	102.3	116	32		45		
18	46	5509	1–12–26	Grp. IV	L. U. L. L. L. L.	3		7	7.40	22.58	43.9	6.13	7.18	85.4	102.4	116	30	Yes			Confluent bronchopneumonia. Death
19	47	5522	2– 1–26	Grp. IV	R. U. L. R. M. L. R. L. L.	12		13	7.36	32.22	65.6	3.70	6.32	58.6	99.4	120	70		40		Death

R. U. L. signifies right upper lobe; R. M. L., right middle lobe; R. L. L., right lower lobe; L. U. L., left upper lobe; L. L. L., left lower lobe.

forty-seven observations were made. The number of observations per individual varied from one to as many as nine. Blood for analysis was drawn by puncture, usually of the femoral artery. The customary precautions were taken against exposure of the blood to air. Analysis of the total carbon dioxide content, oxygen content and capacity, was performed by the method of Van Slyke and Neill (2). The hydrogen ion concentration was measured by the colorimetric method of Hastings and Sendroy (3).

Calculations.

The data presented in Table I and graphically plotted in Fig. 1 were derived, as in the first paper, by calculation from the analyses of

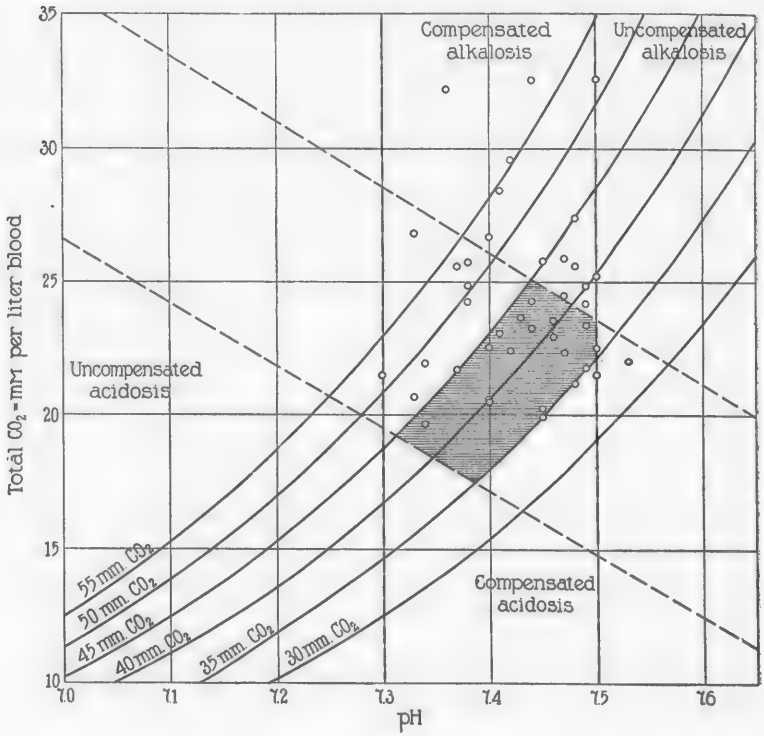

FIG. 1.

pH of serum, and CO_2 content, oxygen content, and oxygen capacity of whole arterial blood.

The fundamental equation used was the familiar Henderson-Hasselbalch formula for whole blood:

(1) $$pH_{t^\circ} = pK_1'_{bt^\circ} + \log \frac{[BHCO_3]}{[H_2CO_3]}$$

$$= pK_1'_{bt^\circ} + \log \frac{[CO_2] - [H_2CO_3]}{[H_2CO_3]}$$

where t° = body temperature in °C., $pK_1'_{bt^\circ}$ = the negative logarithm of the apparent first dissociation constant of carbonic acid in whole blood at body temperature, and the brackets indicate concentrations in terms of mM per liter whole blood.

(2) $$[H_2CO_3] = \frac{[CO_2]}{1 + 10^{pH - pK_1'_{bt^\circ}}}$$

The pH of serum, as usual, was used for whole blood. The value of $pK_1'_b$ was calculated from the equation of Van Slyke, Wu, and McLean (4).

(3) $$pK_1'_b = pK_1'_s + \Delta pK_1'$$

where $pK_1'_s$ stands for pK_1' in serum, and $\Delta pK_1'$ is the correction applied for the difference in pK_1' in whole blood from the serum value. From the pH, oxygen content, and oxygen saturation, $\Delta pK_1'$ was estimated by using the nomogram of Van Slyke, Hastings, Murray, and Sendroy (5). To estimate $pK_1'_{bt^\circ}$, the data of Stadie and Martin (6) were used to calculate the temperature coefficient of this constant. Their values give $\frac{\Delta pK_1'_b}{\Delta t^\circ} = -0.0065$. Hence the complete expression for $pK_1'_{bt^\circ}$ is:

(4) $$pK_1'_{bt^\circ} = pK_1'_{s38^\circ} + \Delta pK_1' \pm \Delta t^\circ\ (-\ 0.0065)$$

where $pK_1'_s = 6.12$, and $\pm t^\circ$ is the difference in °C. between body temperature at the time of bleeding and 38°C.

To estimate the CO_2 tension, the expression:

(5) $$[H_2CO_3] = \frac{\alpha CO_{2_{bt^\circ}} \times pCO_2}{760 \times 0.0224} = 0.0587 \alpha CO_{2_{bt^\circ}} \times pCO_2$$

was used, where $\alpha CO_{2_{bt^\circ}}$ is the solubility coefficient of CO_2 in whole blood at body temperature, and pCO_2 is the CO_2 tension. The value of $\alpha CO_{2_{bt^\circ}}$ being propor-

tional to the water content of the blood. this quantity was calculated as follows. Using equation 30 of Van Slyke, Wu, and McLean (4),

$$(H_2O)_b = 0.914 - 0.015\ (Hb) \tag{6}$$

where $(H_2O)_b$ = kilos H_2O per kilo blood, and Hb = mM Hb per kilo blood as measured by oxygen capacity determinations, the expression:

$$H_2O_b = \frac{0.914\ (Gb) - 0.015\ Hb}{1.007} \tag{7}$$

was derived, where H_2O_b = liters H_2O per liter blood, (Gb) = specific gravity of blood, and Hb = mM per liter blood. (Gb) was calculated from equation 29 of Van Slyke *et al.*:

$$(Gb) = 1.027 - 0.0037\ Hb \tag{8}$$

According to Bohr and Bock, αCO_{2H_2O} at 38° = 0.555 and the temperature coefficient at this temperature is −0.0124.

$$\text{Hence } \alpha CO_{2_{bt°}} = [0.555 \pm \Delta t° (- 0.0124)]\ H_2O_b \tag{9}$$

By combining all of the previous equations, the complete expression for the CO_2 tension is found to be:

$$p CO_2 = \frac{[CO_2]}{0.0583 \times [0.555 \pm \Delta t° (- 0.0124)] \times [0.939 - 0.01162\ Hb] \times \left[1 + 10^{pH - [6.12 + \Delta pK_1' \pm \Delta t° (- 0.0065)]}\right]} \tag{10}$$

Due to the fact that the average Hb concentration and degree of saturation was different from that of the group of cases reported in the first paper, the results, when plotted on the diagram of Hastings, Neill, Morgan, and Binger (1), showed a CO_2 tension different from that calculated. Hence a new diagram was drawn based on calculations of a blood having an average oxygen capacity of 7 mM Hb per liter, and an oxygen saturation of 82 per cent. Calculated CO_2 tensions in most instances agree with those plotted in Fig. 1.

RESULTS.

The results are tabulated in Tables I and II and graphically plotted in Fig. 1, on the modified Van Slyke acid-base diagram.

The pH of the Serum.—Of the 45 observations but one pH was higher than 7.50, all the rest falling within the normal limits of 7.30 to 7.50. The average pH of this series is 7.43. Table II shows a higher frequency of pH on the alkaline side of this normal range.

TABLE II.

Showing the Frequency of Incidence of the Variables Calculated in Table I.

pH	7.30–35	7.36–40	7.41–45	7.46–50	7.51–55			
Incidence	5	11	11	17	1			
CO_2, *mM per liter*	19–21	21.01–22.0	22.01–24.0	24.01–26.0	26.01–28.0	28.01–30.0	30.01–32.0	32.01–34.0
Incidence	5	5	12	14	4	2	0	3
pCO_2, *mm*	30–35	35.1–40.0	40.1–45.0	45.1–50.0	50.1–55.0	55.1–60.0	60.1–65.0	65.1–70.0
Incidence	4	8	14	4	9	1	1	1
O_2 saturation, *per cent*	75 and below	76–80	81–85	86–90	91–95	96–100		
Incidence	12	4	5	7	13	6		

The CO_2 Content of the Blood.—The average of 42 measurements gave a value of 24.26 millimols per liter, which is well within the normal range. Most of our results were clustered about this figure for CO_2 content.

The CO_2 Tension.—Of the 42 CO_2 tensions calculated, half were within the normal limits of 35 to 45 mm., most of the remainder occurring above the higher of these values. The average was 44.4 mm.

The Oxygen Saturation.—The average value for oxygen content was 6.02 mM per liter, and for oxygen capacity 7.12 mM per liter. The average percentage saturation was 84.5 per cent, irrespective of oxygen therapy.

These results, in so far as one may make any inferences under such varying conditions of the disease and its treatment, corroborate, in every respect but one, the observations of the previous paper on this subject. This series fails to show the correlation previously noted, between the CO_2 tension and the patient's temperature. None of the points plotted in Fig. 1 fall in an acidosis area. In fact, those points not in the normal area tend to indicate a condition of compensated alkalosis. The CO_2 content showed little departure from normal values, as has already been noted by various other investigators. The CO_2 tensions, on the other hand, were grouped more in the region of the higher normal values. The oxygen unsaturation in these observations was more marked than that of the first series.

SUMMARY AND CONCLUSIONS.

Estimation of the blood reaction and of blood gases in a series of nineteen individuals suffering from pneumonia failed to reveal a condition of acidosis occurring at any time during the disease. The results of this study are corroborative of a previous one, to which reference has been made.

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STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

II. The Infectivity of Type III Pneumococcus for Rabbits.

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In a previous paper (1) it was shown, in agreement with the experience of others, that the sera of rabbits immunized with Type III pneumococci failed to agglutinate the homologous organisms. Evidence was offered that the failure in agglutination was due, not to the inagglutinable state of the encapsulated bacteria, but to the actual absence of demonstrable type-specific antibodies. However, the sera of the immunized animals were reactive with pneumococcus nucleoprotein regardless of its type derivation and were capable of agglutinating the non-encapsulated degraded variants of all type-specific strains (R forms). This antibody response is contrary to the usual experience in immunization with type-specific pneumococci, when the cells used as antigen are intact. Although the Type III organisms used were, for the most part, possessed of large capsules, the antibody response, instead of being type-specific, was only species-specific in character and simulated that elicited by immunization with pneumococcus protein or with non-encapsulated R forms. The inference drawn from these results was that rabbits possess some mechanism which is capable of affecting the antigenic integrity of Type III pneumococci and that the alteration which the organisms undergo in the animal body is reflected in the character of the antibody response.

Because of the unusual reaction of rabbits to immunization with Type III pneumococci and the implications which these results suggest, investigations have been carried out with regard to the infectivity of this type of pneumococcus for rabbits. The experiments reported in the present paper include observations on the degree of viru-

lence of several strains of Type III, on the character of the bacteremia following intravenous injection, and the relation of phagocytosis to the disposal of the injected organisms.

Although Type III pneumococci are recognized as being highly pathogenic for white mice, reports on their virulence for rabbits have differed. Hanes (2) states that Type III organisms are highly virulent for rabbits without giving dosage or number of strains tested. Singer and Adler (3) found 0.05 to 0.1 cc. of culture usually fatal, but mention the fact that the lethal dose, in their experience, was not constant. They employed two strains and found one more constantly virulent than the other. Lévy-Bruhl (4) found the minimum lethal dose to be 1 cc. with two strains of Type III and greater than 1 cc. with two other strains. Bengtson (5) reported the lethal dose to vary from 0.1 to 1 cc.; whether different strains were tested or not is not stated.

In order to obtain further information concerning the virulence of Type III pneumococci for rabbits, eleven strains of this organism were collected. The strains were obtained from either blood or sputum of patients suffering from lobar pneumonia. All of the strains, which had been recently isolated were found on first injection to be highly virulent for mice. Other strains, which were taken from stock, were first passed through mice to enhance their virulence for these animals before being tested in rabbits. This was done in order to exclude from the cultures degraded R forms of pneumococci, which are non-encapsulated, non-type-specific, and avirulent. Reimann (6) has pointed out that a pneumococcus culture of low virulence may be one in which R forms predominate over type-specific, encapsulated S forms. According to this view, the repeated passage through mice of a culture containing both forms increases the proportion of S organisms until, with the acquisition of maximum virulence, the culture is theoretically composed entirely of type-specific, encapsulated pneumococci. Consequently, the cultures of Type III used for virulence tests in rabbits, by first being made highly pathogenic for mice, fulfilled this requirement.

Rabbits were injected either intravenously or intraperitoneally, with 12 to 14 hour cultures in doses ranging from 2 to 10 cc. It may be seen from Table I that eight of the strains did not produce fatal infection. In the case of two other strains rabbits died following injection of relatively large doses, but the cultures isolated from the

blood of these failed to kill others. Although no sustained effort was made to enhance the virulence of all the strains, most of them were injected several times. Of the eleven strains only one acquired definite virulence for rabbits, which, by repeated passage, finally produced a fatal infection in doses of 0.0001 cc. Even with this strain

TABLE I.

Virulence of Type III Pneumococcus for Rabbits.

Strain of Type III	Virulence for mice	Rabbit No.	Dose of culture	Site of injection	Results
	cc.		cc.		
A	.000001	1	5	Intravenous	Survived
A	.000001	2	2	Intraperitoneal	"
A	.000001	3	2	"	Died—2 days
Blood culture Rabbit 3		4	2	"	Survived
" " "		5	5	"	"
M	.000001	6	2	Intravenous	"
M	.000001	7	3	Intraperitoneal	"
M	.000001	8	10	"	"
F	.000001	9	5	"	Died—3 days
Blood culture Rabbit 9		10	5	"	Survived
F	.000001	11	8	"	"
L	.000001	12	2	Intravenous	"
L	.000001	13	5	Intraperitoneal	"
H	.000001	14	5	Intravenous	"
H	.000001	15	10	Intraperitoneal	"
E	.000001	16	5	"	"
S	.000001	17	5	"	"
S	.000001	18	8	"	"
B3	.000001	19	5	"	"
B3	.000001	20	2	Intravenous	"
B4	.000001	21	5	"	"
B4	.000001	22	5	Intraperitoneal	"
B2	.000001	23	5	"	"
PH*	.000001	24	5	"	Died—2 days

* This strain became highly virulent for rabbits.

the degree of virulence has not remained constant and has often shown evidence of attenuation when kept out of the animal body for several days. Moreover, differences in the natural resistance of individual rabbits to the same strain is, in part, responsible for variations in the degree of virulence.

From the results obtained it seems justifiable to conclude that Type III pneumococci possess only slight initial virulence for rabbits. Furthermore, although type specificity and encapsulation are necessary for the highest degree of mouse virulence, these characteristics are not, in themselves, sufficient to overcome the natural resistance possessed by rabbits.

The conclusion that Type III pneumococci are of low pathogenicity for rabbits was arrived at by the simple procedure of injecting organisms and accepting the ultimate survival or death of the animal as a criterion of virulence. This method, however, throws no light either on the duration and intensity of the infection, or upon the method of recovery, a fact which has been emphasized by Bull (7) in a report on some of the characteristics of streptococcal and pneumococcal bacteremia in rabbits. The technique employed by him consisted in making blood cultures intermittently from the peripheral veins after the injection of organisms. The results obtained by Bull and others (7–10) have demonstrated the reliability of the method. Consequently, since Type III pneumococci failed to produce fatal infections in rabbits, it seemed of interest to observe the course of the bacteremia following injection of these organisms. This was done by means of blood cultures taken at frequent intervals following the introduction of organisms into the circulation. The results are diagrammatically represented in the accompanying text-figures in which the number of colonies per unit of blood is plotted on the ordinates and the time interval, at which the culture was taken, is plotted on the abscissæ (Text-figs. 1, 2, and 3).

There were available for this study both S and R strains of Type III pneumococci. The eleven S strains were typical and biologically identical. They possessed large, easily demonstrable capsules; they grew on blood agar with the production of mucoid colonies; they were bile-soluble; they all reacted equally well in Type III antipneumococcus horse serum. They were pathogenic for humans, the source from which they were derived, and were all equally virulent for mice, killing in doses of 0.000001 cc. However, one of the S strains differed in that it was made virulent for rabbits by rabbit passage, whereas the others were not virulent for these animals in doses ranging from 2 to 10 cc. In addition to the type-specific S strains of pneumococci, non-

type-specific, degraded R forms were used for comparative study. As described by Reimann (6) R forms may be obtained by cultivating type-specific organisms in homologous immune serum. Those used in this experiment were derived from a culture of Type III. Doses of 1 cc. failed to kill mice and doses of 10 cc. failed to kill rabbits; higher doses were not tested. The strains of pneumococci used in the present experiment, then, comprised representatives of each of the three varieties:

1. S strains of Type III pneumococcus; *virulent* for rabbits (designated SV). One strain belonged to this group.

2. S strains of Type III pneumococcus; *avirulent* for rabbits (designated SA). Ten strains belonged to this group.

3. R strains representing the degraded, non-encapsulated, avirulent variants of Type III pneumococci. Bacteria of this character are comparable to non-pathogenic saprophytes.

EXPERIMENTAL.

Cultures.—12 to 14 hour plain broth cultures in standard doses of 2 cc. each were injected intravenously regardless of the strain used. The actual number of organisms per cc. was not determined but under uniform conditions, it may be considered comparable for all the strains.

Blood Culture Technique.—The technique employed varied only in minor details from that described by Bull (7) and was as follows: The organisms were introduced into the marginal vein of one ear of the rabbit and cultures were obtained from the opposite ear. Before making the culture, the ear was closely shaved along the marginal vein and then saturated with 95 per cent alcohol. The alcohol was wiped off with a dry sterile sponge and the vein slit transversely with a razor blade. The blood was allowed to drip perpendicularly from the edge of the ear and, after discarding the first few drops, 6 drops were collected in melted agar kept at 42° to 45°C. The melted agar and blood were mixed rapidly and poured into a Petri dish. The plates were incubated for 36 hours and the number of colonies per plate recorded. Cultures were usually taken 15, 30, and 60 minutes after injection of the organisms, then at 2 hour intervals for 12 hours; after this, 2 or 3 times daily until the animal succumbed or permanent sterility occurred. After the first 12 hours subsequent cultures were taken from blood derived from a fresh slit distal to the previous cuts. This precaution was taken in order to obviate the possibility of organisms resident in the tissues at the site of the previous injury being washed into the agar by the flowing blood.

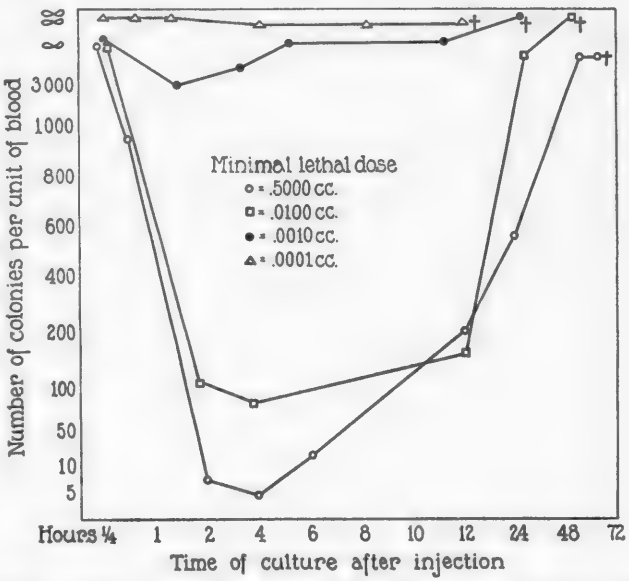
Employing streptococci and pneumococci Bull (7) found that infection in rabbits may take one of three courses depending on the viru-

lence of the bacteria. With highly virulent organisms a rapidly fatal septicemia occurred which was characterized by an initial marked reduction in the number of organisms in the circulating blood, followed by a rapid increase. With less virulent organisms the infection became chronic, the course of the bacteremia was uneven, and, after death, localization of organisms in the serous cavities was often demonstrable. When bacteria of even lower virulence were injected, they quickly disappeared and the blood stream remained sterile. The initial diminution in the number of organisms occurring in experimental septicemia, first described by von Fodor (8), has been repeatedly observed by others. This phenomenon has usually been explained as being due to the dissemination and filtration of the organisms throughout the body tissues. However, that this explanation is not sufficient becomes evident, as will be shown, if the relative virulence of the bacteria injected is taken into consideration.

Course of Bacteremia Following Intravenous Injection of Rabbit Virulent, S Strain of Type III Pneumococcus (SV Strain).

The effect of increased virulence on the course of the bacteremia is shown in Text-fig. 1. These results were obtained by injecting rabbits with a strain of Type III pneumococcus, the virulence of which had been progressively increased by repeated passages through rabbits. The data presented reveal differences in the course of the bacteremia depending on the degree of virulence. When the minimal lethal dose of the rabbit virulent strain (SV) was 0.5 cc. there was a marked initial decrease in the number of organisms in the circulating blood, the minimum occurring 4 hours after infection; then there followed a progressive rise in the number of colonies until the death of the animal occurred 56 hours after injection. This type of curve in experimental septicemia corresponds to the results previously described by others. When the virulence of this same strain had been enhanced so that now, 0.01 cc. of culture proved fatal, the initial decrease was less marked, and the secondary rise more rapid. Injection of a culture of still greater virulence (0.001 cc.) resulted in only a slight initial decrease in the number of organisms in the peripheral blood. When a maximum virulence of 0.0001 cc. had been attained the number of organisms in the blood of the infected rabbit was at no

time decreased and death resulted in 12 hours. In each instance approximately the same number of organisms was injected. Therefore, if the primary decrease represented merely a mechanical process of dissemination and filtration it should occur regularly, regardless of virulence. This, however, was not the case; the extent of the initial decrease in the number of circulating bacteria was in inverse proportion to the degree of virulence (Text-fig. 1).

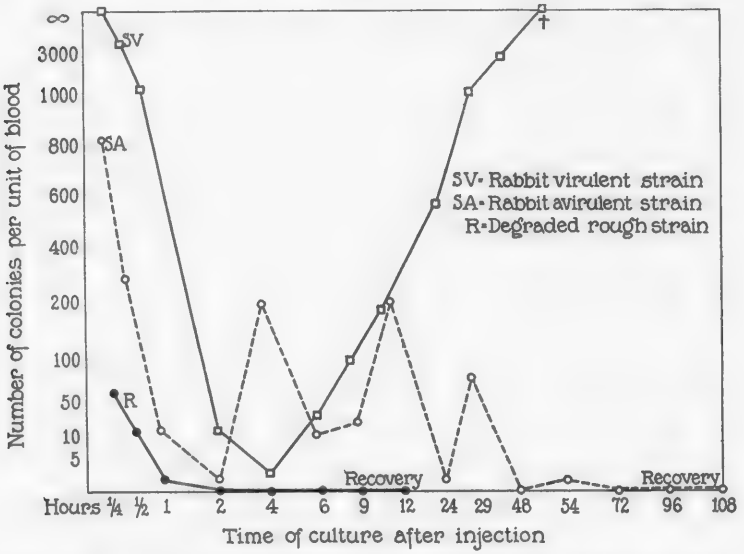


TEXT-FIG. 1. Effect of increased virulence on the course of bacteremia in rabbits injected with Pneumococcus Type III (Strain SV).

In addition to the effect of progressively increased virulence on the course of blood infection caused by the rabbit virulent strain of pneumococcus, certain characteristic differences in the curves were observed when strains of Type III not virulent for rabbits and degraded R forms of pneumococcus were employed. A graphic representation of the course of the bacteremia following injection of organisms of these two varieties is shown in Text-fig. 2. For purposes of comparison the type of curve representing the course of events after injection of the rabbit virulent strain is included in this same experiment.

Course of Bacteremia Following Intravenous Injection of R Strains of Pneumococcus.

From Text-fig. 2 it may be seen that there was an immediate and marked decrease in the number of R organisms in the circulating blood. After 2 hours they completely disappeared and the rabbit remained free from further blood infection.

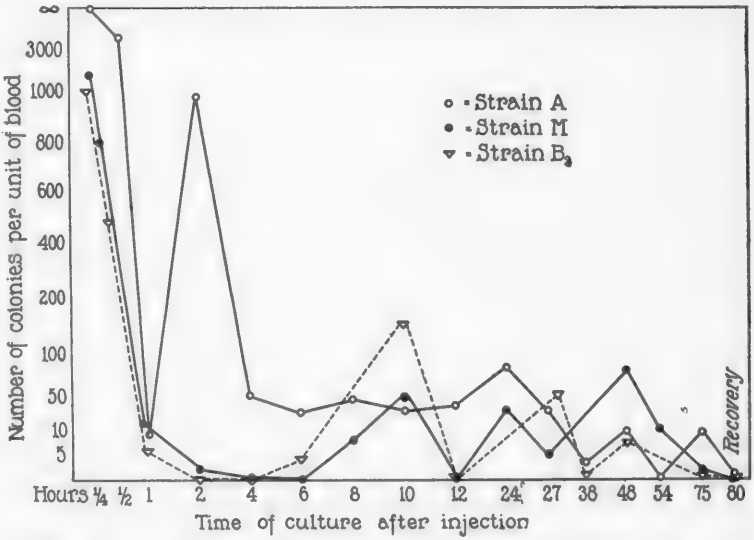


TEXT-FIG. 2. Course of bacteremia in rabbits injected with Pneumococcus Type III (Strains SV, SA, R).

Course of Bacteremia Following Intravenous Injection of Rabbit Avirulent, S Strains of Type III Pneumococcus (SA Strains).

The bacteremia which followed injection of rabbit avirulent strains of Type III pneumococci ran a distinctly different course. From Text-fig. 2 it may be seen that 3 to 4 days elapsed before permanent sterility of the blood was finally attained. While the bacteremia persisted, the chief characteristic was repeated fluctuations in the number of organisms in the circulating blood. At times the cocci entirely disappeared only to reappear again a few hours later. Exami-

nation of the organisms which reappeared failed to reveal any biological differences, when compared with the original cultures. As previously stated, the rabbit avirulent strains (SA) represent all but one of those used in the experiments. Although only one curve characteristic of the bacteremia produced by these strains is shown in Text-fig. 2, repetitions of this experiment gave similar results. In Text-fig. 3 are shown similar curves obtained with three other rabbit avirulent strains.



TEXT-FIG. 3. Course of bacteremia in rabbits injected with strains of Pneumococcus Type III, avirulent for rabbits (Strains SA).

In the experiments just described virulence of Type III pneumococci for rabbits has been considered with special reference to certain properties pertaining to the organisms themselves, such as differences of encapsulation, type specificity, and mouse virulence. These factors have been correlated with the infectivity of the organisms as represented, not only by the ultimate outcome of the infection in rabbits, but also by the character of the bacteremia produced.

In addition to the biological properties of the bacteria, factors related to the resistance of the host require consideration and an investigation of two of these has been carried out.

1. Antibodies.—The sera of normal rabbits have not been found to possess antibodies reactive with the encapsulated Type III cells, or with the soluble specific substance derived from these organisms, or to confer passive protection on mice. Although normal rabbit sera are occasionally encountered which agglutinate R pneumococci, the incidence is not common and the dilutions of serum in which the reaction occurs are low. Whether antibodies of this character are significant in the disposal of R cells after injection into the animal body, is irrelevant to the present study. It can be stated, however, that the phagocytosis of R pneumococci, as described below, is not preceded by agglutination.

2. Phagocytosis.—In studying phagocytosis, the vital staining method described by Sabin (11) for the study of living blood cells has been employed. The technique varied in no detail from Sabin's procedure except for the fact that a small loopful of living pneumococcus culture was added to the blood. The preparations were observed microscopically in a warm chamber kept at 37°C. By this method leucocytes may be watched immediately after removal from the animal body and while actively motile in the environment of whole blood. Preparations of this character approximate conditions existing in the circulating blood and minimize alterations of the leucocytes which may occur in the usual methods of studying phagocytosis *in vitro*. The specimens were observed from 1 to 2 hours. At the end of this time the leucocytes begin to lose their motility and their protoplasm contains large, red staining bodies which Sabin has described as vacuoles. Observations beyond 1 to 2 hours have not revealed phagocytosis which was not present earlier, and the beginning alteration in the leucocytes was evidence that the preparations were not useful for further observation.

When R forms of pneumococci were employed, phagocytosis by polymorphonuclear leucocytes could be seen in 2 to 3 minutes. It continued actively and, after 10 minutes, every leucocyte was engorged with organisms. When phagocytosis is observed under these conditions of vital staining the picture is very striking. Pneumococci, before ingestion, are unstained. However, immediately after being engulfed, they appear as bright red organisms within the body of the leucocyte. The facility with which R cells are phagocyted is

evidence of the prime importance of this activity in the natural resistance of rabbits to blood infection with R forms; the promptness with which these organisms disappear from the blood stream following intravenous injection may be referable, in part at least, to their susceptibility to phagocytosis.

When S forms of Type III pneumococci were mixed with normal rabbit blood, no phagocytosis was observed. This was true whether the encapsulated organisms were virulent or avirulent for rabbits. Occasionally, after an hour a few leucocytes could be found which had ingested one or two bacteria but the picture contrasted sharply with that seen in preparations made with R forms. Since the inability of leucocytes to ingest encapsulated bacteria has been a common observation, and, since this fact has been related to virulence, the failure to demonstrate this correlation in the present study is of special significance. Although phagocytosis as observed *in vitro* may not be identical with the phenomenon *in vivo*, nevertheless, the contrast between the action of leucocytes against R and S forms is striking and justifies the inference that the natural resistance of rabbits to S forms of Type III pneumococci involves factors either additional to or different from phagocytosis.

DISCUSSION.

In the experiments reported in this paper on the infectivity of Type III pneumococci for rabbits certain factors which are known to be associated with virulence and resistance have been taken into consideration. The organisms used in the experiments conformed with certain requirements which might be expected to promote virulence. All of the strains possessed large mucoid capsules and type specificity (S forms). They were obtained from patients suffering from lobar pneumonia and were highly virulent for mice. However, in spite of this, ten out of eleven of these strains failed to produce fatal infection in rabbits when injected in moderately large doses. The other strain was slightly virulent for rabbits on isolation and this property was further enhanced by animal passage. Since no biological differences could be demonstrated between this strain, when possessed of maximum virulence, and the others, it is necessary to assume that viru-

lence in this instance is related to some property not possessed by the other S strains. The course of the fatal septicemia resulting from injection of this strain and alterations in the character of the curve associated with increased virulence are shown in Text-fig. 1.

An attempt to understand the failure of *avirulent, encapsulated* Type III pneumococci (S forms) to cause fatal infection in rabbits led to a study of the fate of these organisms as contrasted with that of *avirulent, non-encapsulated* pneumococci (R forms). Although the ultimate survival of the infected animal occurs in both instances, the bacteremia induced by S forms differs from that induced by R forms. Following injection, the non-encapsulated R cells disappear rapidly and permanently from the blood stream, whereas the avirulent, but encapsulated S forms give rise to a prolonged bacteremia characterized by intermittent increase and reduction in the number of circulating organisms. These differences suggest that the mechanism whereby rabbits overcome the two types of infection is not identical, and that the method of disposal is in some way related to the presence or absence of capsule. Further evidence that the mechanism of recovery in the case of S organisms is different from that effective in disposing of R forms, was brought out in the experiments on phagocytosis. Therefore, it seems obvious that, although the encapsulated state is sufficient to prevent phagocytosis, it is not sufficient to protect the cell against the defense reactions of the host. The resistance of rabbits to Type III pneumococci implies the presence of factors other than phagocytosis. Furthermore, the fact that the sera of normal rabbits do not contain demonstrable type-specific antibodies renders improbable the participation of these immune substances in the mechanism of natural resistance. In a previous paper (1) it was shown that immunization of rabbits with Type III pneumococci failed, in the majority of instances, to stimulate the production of type-specific antibodies, but was effective in producing antibodies reactive against another constituent of pneumococci, namely the nucleoprotein fraction. These experiments indicate that normal rabbits possess a mechanism which is capable of inflicting an injury on the capsule of Type III pneumococci. It has been shown by Avery and Heidelberger (12) that type-specific antibodies are best elicited when S cells in an intact state are used as antigen. They (12) have also

shown that type specificity is intimately related to the soluble specific substance of the capsule of pneumococcus. Therefore, the absence of demonstrable type-specific antibodies in rabbits immunized with encapsulated Type III pneumococci indicates that the animals are capable of damaging the capsular mechanism of these organisms. In the present experiments, the capacity of normal rabbits to inflict injury on Type III pneumococci is further demonstrated by the recovery of the animals following intravenous injection of living S organisms. It seems not unlikely that both the survival of the animal and the altered antigenicity of the cell following injection of the encapsulated organisms are referable to the same mechanism, and upon the factors underlying this defense reaction depends the natural resistance of rabbits to Type III pneumococci.

SUMMARY.

The observations recorded in this paper on the infectivity of Type III pneumococci for rabbits may be summarized as follows:

1. Of eleven strains of Type III isolated from human sources, ten were found to possess low virulence for rabbits. This was true despite the fact that all the strains tested possessed large capsules and a high degree of virulence for mice.

2. One strain of Type III pneumococcus was rendered highly virulent for rabbits. Since it possessed no other biological property demonstrably different from the other strains, its virulence must reside in some additional property.

3. An initial decrease in the number of circulating organisms following the injection of virulent bacteria is a well known occurrence, and it was observed in rabbits injected with the rabbit virulent strain of Type III. However, the extent of the reduction was in inverse proportion to the degree of virulence of the strain; a fact which makes mechanical explanations of the phenomenon insufficient.

4. The bacteremia produced in rabbits by Type III pneumococci, avirulent for this species, runs a characteristic course. It differs from that produced by non-encapsulated R forms of pneumococci although in both instances survival of the infected animal ensues. This is evidence that the mechanism of resistance against encapsulated and non-encapsulated pneumococci is not identical.

5. Phagocytosis of Type III pneumococci by circulating rabbit leucocytes was not demonstrable by a vital stain technique, whereas under the same conditions the ingestion of non-encapsulated R forms occurred. This is further evidence that the process whereby non-encapsulated pneumococci are disposed of, is insufficient to explain the natural resistance of rabbits to infection with encapsulated Type III pneumococci.

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